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BIOCHEMISTRY OF MYOGLOBIN. VII. THE EFFECT OF COOKING ON MYOGLOBIN IN BEEF MUSCLE[a]

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(Manuscript received August 22, 1958)

Among the chemical changes that accompany the cooking of fresh beef are color changes which reflect alterations in the state of the muscle pigment myoglobin as it is affected by heat. The pigments of cooked fresh meat are of importance both because color is universally used as a guide to the degree of doneness and because heme compounds have been implicated as catalysts for certain undesirable reactions in cooked meat *(8)*.

Beef cooked to an internal temperature of 60° C. or less is considered rare and has a bright red interior; beef cooked to an internal temperature of 60° C.–70° C. is considered medium done and has a pink interior; beef cooked to an internal temperature of 70° C.–80° C. or higher is considered well done and is grey-brown throughout *(1, 4, 6)*. During the process of cooking meat to a certain internal temperature, the surface naturally receives the greatest amount of heat for the longest period of time while the interior receives the least heat for the shortest time, with the result that the state of the pigment as it is altered by heat would be expected to vary continuously from the exterior to the interior. Cooking conditions, too, may further affect the apparent degree of doneness. Cover, Bannister, and Kehlenbrink *(2)*, reported that braised steaks cooked over boiling water to an internal temperature of 85° C. were underdone and the meat still pink. The pink pigments occurring in cooked beef are the subject of the present investigation.

On the basis of reflectance spectra, Tappel *(7)*, concluded that the pink pigments lying adjacent to the brown pigments in underdone cooked beef consist of undenatured oxymyoglobin. The insoluble brown pigments of cooked meat have been recognized as denatured globin ferric hemichromogen *(9)*. If the pink pigments found in cooked beef do consist of undenatured oxymyoglobin, then it should be possible to extract them with water. In the present paper, the spectral and ultracentrifugal characteristics of the pigments extracted from cooked beef have been examined, and the results have been found to substantiate the findings of Tappel *(7)*. Further work has also been done to determine the effect of heat upon the denaturation of myoglobin in meat. The water extractability of the pigments in the cooked samples was used as a measure of the degree of pigment denaturation.

EXPERIMENTAL

Cooking. For obtaining the pink cooked pigments, flank steak which was purchased at a local supermarket and then finely ground was packed into a shallow pyrex

[a] This paper has been assigned Journal Paper number 168 American Meat Institute Foundation.

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tray in a layer ½-in. thick and cooked uncovered in a preheated forced-air oven at 80° C. for 2 hr. At that time, the internal temperature as measured with a mercury thermometer was 60° C. After cooking, the meat was inverted in the tray, cooled to 5° C. in a cold room at that temperature and then blended with 150 ml. distilled water per 100 gm. wet tissue for 1 min. in a Waring blender. An uncooked control was similarly extracted. The homogenates were centrifuged, and absorption spectra were taken of the filtered supernatants.

Spectrophotometry. Absorption spectra were taken with a Beckmann DK recording spectrophotometer. Concentrations were determined with a 1.00 cm. cuvette from the extinction at 525 mu. (EmM = 7.3), which is an isobestic point for all the soluble myoglobin derivatives mentioned in this paper.

Ultracentrifugation. Sedimentation measurements of the pigments extracted from cooked and uncooked flank steak were made using a Spinco model E ultracentrifuge. In calculating the $S_{20,w}$ for myoglobin, a partial specific volume of 0.741 was assumed, and the density and viscosity of the supernatants remaining after deproteinization by boiling for 1 hr. were used. Cooked and uncooked samples were sedimented both as the crude extracts and after extensive dialysis against distilled water. The values reported are averages.

Denaturation. Heat denaturation curves for myoglobin in cooked beef were determined on 3 separate occasions for every temperature investigated, and for each determination the meat samples consisted of three individual flank steaks which were ground and uniformly mixed. The values reported are averages. In these studies, 10 g. samples of meat were packed into the bottoms of 50 ml. polyethylene centrifuge tubes which were then covered with aluminum foil and heated for various lengths of time in a temperature regulated water bath. The time required for thermal equilibrium of the samples (10–20 min.), was measured, and the rate of cooling was adjusted to compensate for this. After cooling to 5° C., each sample was extracted overnight at 0° C. with 25 ml. distilled water, and was then centrifuged at the same temperature. The supernatants were filtered twice through coarse and fine filter paper, and the absorption spectra were taken. The per cent denaturation was determined from the spectral data.

Comparable heat denaturation studies were also carried out on a smaller scale with pork. Three medium sized rolled pork roasts were ground and uniformly mixed, and 10 gm. samples were packed into the bottoms of 25 ml. pyrex culture tubes. The samples were cooked, extracted, and analyzed in the manner described above, except that 20 ml. of distilled water was used, and the cooled samples were transferred to centrifuge tubes before extraction. Duplicates were run for each temperature studied, and the averages are given.

RESULTS AND DISCUSSION

Heating the flank steak in the forced-air oven caused surface desiccation and the formation of a crust which assumed a deep red color. After removal from the oven and inversion, the purple-grey color of the cooked meat changed to red upon cooling. The water loss on cooking was 10%, and comparison with the uncooked control showed that 60% of the total water soluble pigments had denatured. From spectral ratios of oxymyoglobin and metmyoglobin, it was determined that the aqueous extract of the cooked meat had an absorption spectrum of 95% oxymyoglobin and 5% metmyoglobin, compared with 80% oxymyoglobin and 20% metmyoglobin for the uncooked control. Treatment with ferricyanide changed the spectrum of the cooked meat extract to that of metmyoglobin which upon reduction with dithionite changed to the spectrum of reduced myoglobin. The spectra obtained from this experiment are given in Figure 1.

The observation that nearly all of the soluble pigments in the cooked beef are extracted as oxymyoglobin indicates that conditions develop in the meat upon cooking which reduce metmyoglobin to myoglobin having

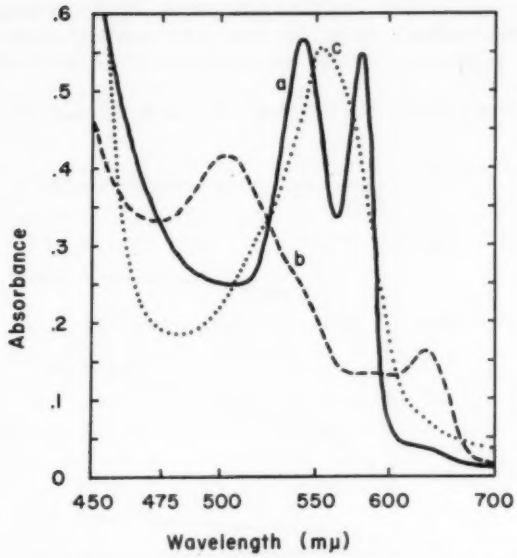

Figure 1. a, absorption spectrum of aqueous extract of ground flank steak cooked to an internal temperature of 60° C.; b, after treatment with ferricyanide; c, curve obtained from a solution of curve b after treatment with dithionite.

the purple-grey color noted, or that the metmyoglobin is more rapidly denatured than the reduced pigment.

Although the spectrum of the pigment was an unaltered spectrum of oxymyoglobin, the possibility still existed that the protein moiety was not intact. Further information was obtained from sedimentation studies. In view of the complex composition of the beef muscle extracts and the concomitant loss of significance of the sedimentation measurements, only the most general conclusions may be drawn from the data listed in Table 1.

The agreement between the values in Table 1 shows that there is little if any alteration of the globin moiety of the heme pigment. This evidence plus the spectral-chemical behavior support the conclusion that the red pigment is undenatured oxymyoglobin. Since the fresh extract contains a relatively high concentration of proteins, it is possible that the large sedimentation constant found under these conditions is the result of the association of myoglobin with other proteins, thus causing an apparent increase

TABLE 1

Sedimentation constants for various myoglobin preparations

Myoglobin preparation	$S_{20,w}$
Extract of fresh flank steak (average of 3 runs)	2.04 ± .14S
Extract of cooked flank steak (average of 2 runs)	1.76 ± .01S
Crystalline beef metmyoglobin *(5)*	1.78S

in molecular weight. The fact that a relatively large percentage of myoglobin still remained soluble in the meat after cooking made it desirable to determine more precisely the denaturation of the pigment at various temperatures.

Data for the denaturation of myoglobin upon cooking are summarized

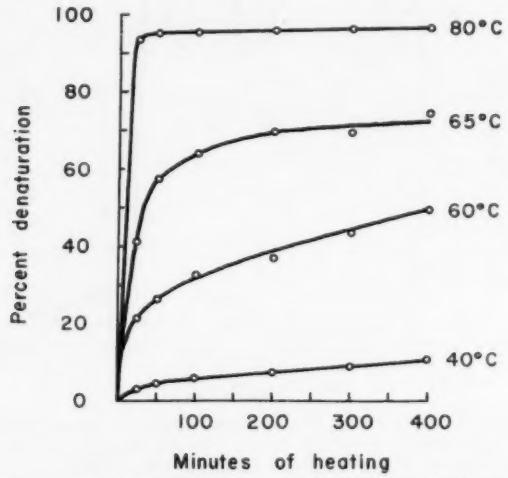

Figure 2. The per cent denaturation of myoglobin in flank steak as measured from the water extractability of the pigment in 10 gm. samples cooked at various temperatures.

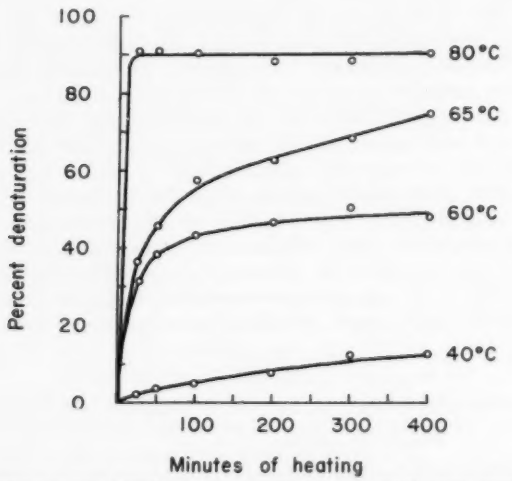

Figure 3. The per cent denaturation of myoglobin in rolled pork roast as measured from the water extractability of the pigment in 10 gm. samples cooked at various temperatures.

in Figures 2 and 3 for flank steak and rolled pork roast, respectively. In all cases, the pigments extracted from the cooked samples consisted mainly of oxymyoglobin. The figures show that the percentages of pigment denatured on cooking are approximately the same in both beef and pork. At 80° C., the denaturation may be accounted for by the thermal denaturation of the pigment, however, at temperatures of 65° C. and below, the denaturation of myoglobin in meat is considerable and cannot be accounted for by thermal denaturation alone, inasmuch as in pure solution denaturation is not appreciable below 65° C. *(3)*. In the case of cooked meat, pigment denaturation at these temperatures may be the result of enzyme action or coprecipitation.

SUMMARY

Pigments extractable from cooked fresh beef consist mainly of oxymyoglobin. The amount of pigment remaining undenatured in a piece of cooked meat depends upon its temperature and how long it has been held at that temperature. The denaturation curves for myoglobin in cooked flank steak have been determined and compared with the pigment denaturation curves in cooked rolled pork roast. Denaturation of myoglobin in meat is considerable at temperatures at which the thermal denaturation of the pigment is negligible in pure solution.

Acknowledgment

The authors are grateful to Dr. B. M. Watts, F. C. Vibrans Fellow at the American Meat Institute Foundation, 1958, for her helpful suggestions.

LITERATURE CITED

1. Committee on Preparation Factors, National Cooperative Meat Investigations. *Meat and Meat Cookery*. 1942. National Livestock and Meat Board, Chicago, Illinois.
2. Cover, S., Bannister, J. A., and Kehlenbrink, E. Effect of four conditions of cooking on the eating quality of two cuts of beef. *Food Research*, **22**, 635 (1957).
3. Fox, J. B., Jr., and Schweigert, B. S. The thermal denaturation of beef myoglobin. (in preparation.)
4. Jensen, L. B. *Meat and Meat Foods*. 1949. Ronald Press, New York.
5. Lewis, U. J., and Schweigert, B. S. Homogeneity studies with crystalline beef myoglobin. *J. Biol. Chem.*, **214**, 647 (1955).
6. Lowe, B. *Experimental Cookery*. 1955. John Wiley & Sons, New York.
7. Tappel, A. L. Reflectance spectral studies of the hematin pigments of cooked beef. *Food Research*, **22**, 404 (1957).
8. Tims, M. J., and Watts, B. M. Relationship of meat pigments to fat oxidation. Abstracts, Institute of Food Technologists, *Food Technol.*, **12**, 46 (1958).
9. Watts, B. M. Oxidative rancidity and discoloration in meat. *Adv. Food Research*, **5**, 1 (1954).

NIACIN CONTENT OF COFFEE IN CENTRAL AMERICA[a]

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(Manuscript received August 5, 1958)

Roasting of coffee beans produces a large increase in their niacin content *(1, 4, 12, 13, 19, 20)*. This niacin is readily extractable with water so that microbiological and chemical analysis of coffee beverage shows it to have a significant niacin content *(19, 20)*. Animal trials *(19, 20)* and metabolic studies in adults *(11)* have confirmed the biological activity of this niacin and shown it to be as available as the niacin in foods and vitamin preparations.

Although high corn diets have frequently been associated with niacin deficiency and clinical pellagra *(5)* this does not appear to be the case for Mexico and Central America where corn is the main dietary staple *(4, 6, 7, 8, 9, 10, 15, 16, 17, 18)*. Nutritional surveys carried out in Guatemala have shown that in adults the total niacin intake is slightly above INCAP recommended allowances *(14)* even without considering the possible contribution of coffee *(6–10)*. Corn contributes about 67.7%, black beans about 10.5% and meat 14.2% of the daily niacin intake *(15, 16, 17, 18)*. In general, all nutritional surveys carried out in the Central American countries *(6–10, 16, 17, 18)* have indicated that niacin is not a nutritional problem in this area for the adult population *(6–10)*, although the niacin intake of pre-school children may be low *(10)*.

Coffee consumption is high in Central America, even in young children, although quantitative data are not available. The amounts of niacin consumed in coffee were considered of possible nutritional importance. Since niacin intakes appear to be borderline in some instances *(6–10)*, the present work investigates the effect of roasting and preparation procedures on the niacin content of coffee samples in Central America and the consumption of coffee as a beverage.

MATERIAL AND METHODS

The roasted coffee bean samples used in the study were purchased at retail in Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica and Panama and represented crops grown in each of these countries. Moisture, nitrogen and ether extract determinations were carried out according to the methods of the A.O.A.C. *(2)*. Niacin was determined microbiologically with *Lactobacillus arabinosus* 17-5 as the test organism *(21)*.

EXPERIMENTAL PROCEDURES AND RESULTS

Niacin content of roasted coffee. Twenty-two samples of Guatemalan coffee, having an average moisture content of 5.48% (range, 3.49 to 8.39%), had an average niacin content of 12.79 mg. % (range, 4.40 to 24.62 mg. %). A group of 6 coffee samples from El Salvador averaged 6.66% moisture (range, 5.37 to 8.15 %), with an average of 18.93% niacin content (range, 14.45 to 25.90 mg. %). The 5 samples obtained from Honduras averaged 5.97% moisture (range, 4.72 to 8.00%) with an average nicotinic acid content of 16.61 mg. % (range, 7.50 to 24.20 mg. %). The moisture content of 11 roasted coffee samples from Nicaragua averaged 5.68% (range, 5.11 to 6.43%) and

[a] INCAP Publication I-115.

the average niacin content was 12.07 mg. % (range, 6.85 to 16.75 mg. %). Five samples of Costa Rican coffee, having an average moisture content of 6.47 (range, 5.77 to 7.12%), had an average niacin content of 17.34 mg. % (range, 12.20 to 21.08 mg. %). The 4 samples obtained from Panama averaged 6.83% moisture (range, 5.37 to 8.25%), with an average niacin content of 22.15 mg. % (range, 18.60 to 24.00 mg. %).

Niacin content of green coffee. The niacin content of samples of raw coffee beans from different coffee plantations in Guatemala is shown in Table 1. These green coffee beans varied in niacin content from 0.60 to 1.33 mg. per 100 g.

TABLE 1

Moisture and nicotinic acid content of Guatemalan green coffee samples

Coffee variety or type	Moisture	Nicotinic Acid
	%	*mg./100 g.*
Robusta	9.50	1.23
Arábigo—1	9.26	1.02
Arábigo—2	10.50	1.21
Arábigo—3	9.19	1.13
Arábigo—4	4.85	1.18
Pache	10.53	0.60
Bourbon	10.21	0.60
Maragogipe	9.94	1.24
Fair washed	9.35	1.07
Extra prime	8.82	1.23
Strictly hard bean	9.40	1.33
Average	9.23	1.08

Effect of temperature, time and type of coffee on the niacin content upon roasting. Three types of coffee were used for this part of the study. A *strictly hard bean* type cultivated at 5,000 feet, *extra prime,* type which comes from plantations of 3,500 feet and a *fair washed* type cultivated at 1,500 feet. Optimum temperatures and times vary with each type of coffee-type. For the experiments, 1½ pound samples were placed in an electrically heated roaster, already set at the desired temperature, and roasted at 3 different times for each type of coffee and temperature. The roasted coffee was ground, and moisture, ether extract and niacin determined. As shown in Table 2, lengthening the time of roasting increased the amount of niacin in the roasted coffee. The niacin content of the *fair washed* type of coffee increased 13 times its original value after 12 minutes of roasting at 180° C. The increase for the *extra prime* type of coffee was the smallest, amounting to about 5 times the original value in 16 minutes at 210° C., while the niacin content of the *strictly hard bean* type increased about 4 times in 6 minutes and about 24 times in 8 minutes. This latter sample was considered already burned and would not ordinarily be used for the preparation of the beverage.

The ether extractable substances also increased with increasing time of roasting. There was a 2.5 fold increase in the ether extract of the *fair washed* type of coffee after 12 minutes of roasting at 180° C. The increase for the *extra prime* type was not as large, being only about 1.7 times its original value in 16 minutes at a temperature of 210° C. In 8 minutes at a temperature of 275° C. the *strictly hard bean* type increased in crude fat content about 2.3 times its original value of 7.65%. The moisture content decreased with increasing time of roasting for all types of coffee studied. In order to study the changes taking place in the weight, moisture, ether extract and nicotinic acid content of green coffee, 200 g. batches of *extra prime* coffee were roasted for 4, 5, 6, and 10 minutes at approximately 300° C. After roasting the sample was allowed to cool to room temperature and weighed; sub-samples were then withdrawn for the analyses and the results are shown in Table 3. The weight of the roasted coffee decreased rapidly before reaching 5 minutes of roasting but did not change significantly thereafter. The moisture decreased rapidly upon roasting, the decrease having been progressive with the duration of the process. These changes in water content upon roasting are not entirely due to loss of water, since the process gives rise to the production of volatile compounds such as carbon dioxide and others which would be decomposition products

TABLE 2

Effect of temperature, time and type of coffee on the nicotinic acid and ether extract content of coffee upon roasting

Time in minutes	Moisture	Ether Extract	Nicotinic Acid
Fair Washed Roasting Temperature: 180° C.			
	%	%	*mg./100 g.*
0	9.35	6.56	1.07
7	3.49	9.61	3.93
9	3.13	14.46	10.00
12	0.15	16.35	13.30
Extra Prime Roasting Temperature: 210° C.			
0	8.82	6.91	1.23
11	2.35	9.66	3.15
14	2.14	8.86	4.40
16	0.50	11.66	5.65
Strictly Hard Bean Roasting Temperature: 275° C.			
0	9.40	7.65	1.33
5	3.82	9.51	2.65
6	2.03	14.15	5.93
8	1.50	17.55	32.66

of carbohydrates and other organic compounds. Both the ether extract portion and the amount of niacin increased on a percentage and on an absolute basis up to 4 minutes of roasting. The finding of a large increase in the ether extract is unusual and warrants further investigation.

Nicotinic acid extraction by percolation. For the percolation studies, 10 g. of a low (7.75 mg. per 100 g.), an intermediate (13.66 mg. per 100 g.) and a high (20.64 mg. per 100 g.) niacin content coffee sample were ground to approximately 40 mesh and percolated in a 4-cup capacity aluminum percolator, heated on a hot plate, with 250 ml. of water for 3, 6, 9, 12 and 15 minutes after the water started boiling. The volume of the infusion was measured after cooling and diluted to 250 ml. with distilled water. The pH of the solution was measured with a Beckman pH meter.

Under the conditions of the experiment, 75% of the niacin in the low niacin sample was extracted. There was no relation to percolation time since niacin extraction was nearly as great after 3 as after 15 minutes of percolation. The percentage extraction for

TABLE 3

Effect of roasting time on the ether extract and nicotinic acid content of roasted coffee

Roasting time in minutes	Wt. of roasted coffee	Moisture	Ether extract		Nicotinic acid	
				Total [1]		Total [1]
	g.	*g.%*	*g.%*	*g.*	*mg.%*	*mg.*
0	200.0	7.84	6.88	14.93	1.25	2.71
4	169.1	1.75	14.58	25.09	14.20	24.44
5	110.8	1.05	19.60	21.95	19.70	22.06
6	110.4	1.52	19.30	21.64	19.30	21.64
10	117.8	0.80	18.20	21.61	21.65	25.73

[1] Ether extract and nicotinic acid content of total sample on a moisture-free basis.

the intermediate niacin coffee varied from 95 to 111%, in a manner unrelated to length of percolation. The high nicotinic acid containing coffee sample gave 52% extraction at the end of 3 minutes of percolation, the extraction increasing to slightly over 100% with increasing percolation time. The highest percentage obtained was 120%, when the coffee was percolated for 15 minutes. The niacin recoveries over 100% might be due either to error in the nicotinic acid assay technique, or more likely to the possible conversion of trigonelline into nicotinic acid during the percolating process.

NIACIN EXTRACTION BY BOILING AND DRIPPING

Boiling. Ten-gram samples of a low (9.40 mg. per 100 g.), an intermediate (16.35 mg. per 100 g.) and a high (25.40 mg. per 100 g.) niacin containing roasted coffees were added to 250 ml. of boiling water. The slurry was then boiled for 2 minutes, filtered, cooled and its volume and pH measured. The preparation was brought to pH 6.8 and a volume of 250 ml. before the niacin assays. The low nicotinic acid containing coffee sample gave 73% extraction of its niacin, while the intermediate and high nicotinic acid coffees gave 82 and 84% extraction, respectively. Nicotinic acid recoveries were 86, 98 and 99% for the low, intermediate and high niacin samples, respectively.

Dripping. A ten-gram sample of each of the 3 roasted coffee samples used in boiling studies was placed on a funnel with a filter paper and washed continuously 16 times with 15 ml. portions of boiling water, in such a way that there was a constant flow of water through the sample. The pH was measured and adjusted to 6.8 and the volume brought to 250 ml. The low niacin containing coffee gave 93% extraction of its niacin, while the intermediate and high nicotinic acid containing coffees gave 84 and 89% respectively. Vitamin recoveries were 100% for the low nicotinic acid containing coffee, 91 and 99% for the intermediate and low coffee samples, respectively.

To investigate if increasing the boiling time would increase the amount of vitamin in the infusion, a 10-g. sample of coffee containing 9.40 mg. of niacin per 100 g. was boiled with 250 ml. for niacin assay. The residue was hydrolyzed and its vitamin content determined.

The results indicate that there appears to be an increase in percentage extraction with boiling time from 72% at the end of 2 minutes to 91% at the end of 6 minutes. Nicotinic acid recoveries varied from 85% at 2 minutes boiling time to 112% at the end of 6 minutes. At the same time, the effect of the amount of sample used on the amount of niacin in the infusion when boiling time and volume were constant was studied by boiling 5, 10, 15 and 20 g. with 250 ml. of water for 2 minutes. Niacin was also determined on the individual residues. Highest extraction and recovery was obtained when 15 g. of coffee were boiled. With this amount the percentage extraction amounted to 94%, while the nicotinic acid recovery was 106%.

Urban coffee consumption and niacin intake. A sample of both the roasted coffee and of the beverage from the morning meal was obtained from 21 persons working in the Institute of Nutrition of Central America and Panama (INCAP). One beverage sample was prepared by the drip method while seven were prepared by boiling, and 13 by percolation as shown in Table 4. The 21 roasted coffee samples averaged 14.31 mg. with a variation of 7.69 to 25.45 mg. of niacin per 100 g. The beverage ranged in niacin content from 0.34 to 2.85 mg. per cup, with an average of 1.03 mg. per cup (175 ml.). The number of cups consumed per person per day ranged from 1 to 8 and averaged 3.3 cups. The niacin intake, therefore, ranged from 0.3 to 16.9 mg. with an average of about 4 mg. Thus the niacin intake varied from 2% to 125% of the INCAP recommended allowances for the Central American area *(14)*.

Coffee consumption in Guatemalan rural villages. Three rural villages in the highlands in Guatemala were chosen for the survey. From 114 to 192 homes per town (representing approximately 2/3 of the population in each case) were visited to determine the brand, number of cups and method of preparation of coffee. In general, it was found that the families bought either coffee blends known to contain roasted cereal grains or brands not common to the city markets. In the town San Lorenzo el Cubo, whose people live next to highland coffee plantations, 55% of the families interviewed consumed coffee harvested and roasted at home, while about 32% of the families consumed a coffee brand whose name indicated it was not pure coffee. The rest of the families consumed little known coffee brands. Of the total number of families interviewed, samples of the coffee and beverage were obtained, representing equal percentages of each

TABLE 4

Nicotinic acid intakes from coffee by persons at INCAP

Method of preparation	Niacin in coffee	pH	Niacin per cup	Cups per day	Niacin intake	Niacin intake % of requirement [1]
	mg./100 g.		*mg.*		*mg./day*	
B [2]	24.62	5.8	2.85	3	8.55	63
P [3]	14.74	6.0	1.23	3	3.69	27
P	20.70	5.6	2.11	8	16.88	125
P	17.45	5.5	1.99	3	5.97	44
P	14.30	5.1	0.83	2	1.66	12
B	13.70	5.6	0.41	3	1.23	9
B	14.45	5.7	0.62	3	1.86	14
P	7.75	5.2	1.19	3	3.57	26
P	9.72	5.6	0.65	6	3.90	29
D [4]	9.98	5.3	0.48	5	2.40	18
P	25.45	6.3	0.83	2	1.66	12
P	13.68	5.5	0.95	3	2.85	21
P	8.27	5.3	0.36	3	1.08	8
P	12.92	5.0	0.84	5	4.20	31
P	13.55	5.3	0.96	3	2.88	21
B	13.40	5.4	1.11	2	2.22	16
B	18.72	4.8	0.64	2	1.28	9
P	15.15	4.9	0.34	1	0.34	2
B	11.40	5.8	0.41	2	0.82	6
P	7.69	5.9	0.92	4	3.68	27
B	12.82	5.0	1.95	4	7.80	58
Average	14.31	—	1.03	3	3.74	28

[1] For the adult in the Central American area, the nicotinic acid requirement is around 13.5 mg./day *(14)*.
[2] Boiling; [3] Percolation; 4 Dripping.

brand of coffee used and the results are shown in Table 5. In Magdalena Milpas Altas the nicotinic acid content of the coffee samples varied from 7.38 to 22.96 mg. per 100 g. and the volume of the cup used varied from 175 to 240 ml. The niacin content per cup varied from 0.24 to 1.46 mg., and an average of 3 cups per person per day were consumed. The niacin intake per person per day from coffee alone varied from 0.72 to 4.38 mg. equivalent to 5 and 32% of the recommended allowance, respectively. In Santa María Cauqué, the nicotinic acid content of the coffee varied from 10.28 to 22.98 mg. per 100 g. The volume of the cup used by the families varied from 150 to 360 ml. The niacin content per cup ranged from 0.20 to 1.90 mg., while the number of cups per day varied from 2 to 4, mostly 3. The niacin intake per day ranged from 0.60 to 6.76 mg., corresponding to 4 and 50% of the nicotinic acid requirement for this area. Finally, in San Lorenzo el Cubo, the 9 samples studied ranged in nicotinic acid content from 4.82 to 43.87 mg. per 100 g. The volume of the cup was smaller than in the two other towns, all of them of 180 ml., except in one case, which was 150 ml. Of the 9 families interviewed, in five the number of cups per person per day was 3, while 4 families consumed an average of 2 cups per person per day. According to this, the niacin intake varied from 0.54 mg., 4% of the requirement, to 1.46 mg. equal to 11% of the recommended allowance for this Guatemalan area.

DISCUSSION

The results of the studies reported in this paper agree with the results of other investigators *(4, 12, 13, 19, 20)* who have reported a marked increase in the niacin of coffee during the roasting process. The variation in niacin content, however, was found greater in the retail roasted coffee bean samples studied, than reported by Gross Daum *(12)* in Venezuela. In general, the nicotinic acid content of roasted coffee sold at retail in

the Central American countries is lower than that reported by other investigators, probably because light-roasting of the coffee is preferred in most cases. Exceptions to this were found in the coffees from El Salvador and from Panama.

As was also found by Teply, Krehl and Elvehjem *(19)*, Teply and Prier *(20)* and Gross Daum *(12)*, the niacin is easily extracted regardless of the method of coffee preparation, since percolation, dripping, or boiling gave over 71% extraction of the niacin present.

TABLE 5

Coffee consumption and niacin intake in three rural villages in Guatemala

Family	Niacin in coffee	pH	Ml. per cup	Niacin per cup	Cups per day	Niacin intake	Niacin intake % of requirement
	Magdalena Milpas Altas						
	mg./100 g.			*mg.*		*mg./day*	
1	13.86	6.8	240	0.38	3	1.14	8
2	8.92	7.3	240	1.46	3	4.38	32
3	9.50	5.8	240	0.64	2	1.28	9
4	7.80	6.3	180	0.28	3	0.84	6
5	7.38	5.8	240	0.26	3	0.78	6
6	12.94	6.0	240	1.00	3	3.00	22
7	9.40	5.6	175	0.73	3	2.19	16
8	12.86	6.4	240	0.38	3	1.14	8
9	18.74	5.9	240	0.56	3	1.68	12
10	22.96	6.5	240	0.24	3	0.72	5
Average	12.44		228	0.59	3	1.72	12
	Santa María Cauqué						
1	22.70	5.5	150	0.71	4	2.84	21
2	11.26	6.3	180	0.65	3	1.95	14
3	13.20	6.0	360	0.44	2	0.88	6
4	18.47	5.8	360	0.56	2	1.12	8
5	21.98	6.2	180	1.69	4	6.76	50
6	10.28	6.2	180	0.20	3	0.60	4
7	20.48	5.9	360	1.90	3	5.70	42
8	22.98	6.1	180	0.39	3	1.17	9
9	14.28	7.2	240	0.42	3	1.26	9
Average	17.29		243	0.77	3	2.48	18
	San Lorenzo el Cubo						
1	14.34	6.3	180	0.45	3	1.35	10
2	7.28	6.4	180	0.29	2	0.58	4
3	8.38	6.5	180	0.33	2	0.66	5
4	43.87	7.7	180	0.73	2	1.46	11
5	29.30	6.7	150	0.33	3	0.99	7
6	13.56	6.9	180	0.37	3	1.11	8
7	34.53	7.0	180	0.27	2	0.54	4
8	4.82	7.6	180	0.19	3	0.57	4
9	5.17	6.6	180	0.18	3	0.54	4
Average	17.92		177	0.35	3	0.87	6

Besides degree of roast, the type of coffee roasted seems to be of importance in determining the amount of niacin produced. However, no definite conclusions can be drawn from the studies presented here, because temperature was also a variable factor. Variety and processing of the cherry could also be of importance in determining the amount of niacin produced under standardized conditions of roasting, although Hughes and Smith *(13)* reported that the nicotinic acid content of raw coffee is not dependent on variety. The content of trigonelline, however, could be dependent upon variety, altitude, or the cherry processing method and thus determine the niacin content of the roasted product, since trigonelline is the substance which upon heat is changed into nicotinic acid *(12, 20)*.

Green coffee samples varied greatly in niacin content, except for two samples, type "Pache" and type "Bourbon," which contained significantly lower amounts of the vitamin. Since most of the samples came from plantations of altitudes over 3,500 feet over sea level, and were cultivated under very similar conditions, these differences must be primarily due to variety. The amount of ether extractable substances also increases with the time of roasting, due partly to the decrease in moisture with roasting and partly to the loss in weight from a known amount of green coffee. However, other changes are taking place in compounds which become ether soluble upon heat. Since this increase in ether extractable substances may be of importance in determining the flavor of the coffee, it should be studied further.

The surveys of the importance of coffee as a contributor of nicotinic acid indicate that significant quantities of the vitamin are ingested in this beverage although the amount varies with the degree of roasting and the strength of the beverage as well as the number of cups consumed per day. Nevertheless for the people who need the vitamin the most, the amount of niacin ingested by drinking coffee is usually relatively small. Because the cost of the roasted products is relatively high, low-income groups frequently cannot afford to buy pure coffee and purchase instead brands that contain large amounts of foreign materials, especially cereal grains and dried beans. Even though the total niacin intake from coffee in the low-income population of Central America is not high, it should be taken into consideration when carrying out and computing dietary surveys.

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LITERATURE CITED

1. Adamo, G. Il contenuto in acido nicotinico nel caffe. *Bol. della Societa Italiana di Biologia Sperimentale*, 31, 79 (1955).
2. Assoc. Offic. Agr. Chemists. *Official Methods of Analysis*. 8th. ed. 1955. Washington, D. C.
3. Castillo, A. S., and Flores, M. Estudios dietéticos en El Salvador. II. Cantón Platanillos, Municipio de Quezaltepeque, Departamento de La Libertad. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*. "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 54-65 1955.

4. Cravioto, R. O., Guzman, G. J. and Suarez, M. L. Incremento del contenido de niacina (vitamina antipelagrosa) durante la torrefacción del café y su significado en el aporte de esta vitamina a la dieta. *Ciencia* (Mexico), **15**, 24 (1955).
5. Food and Agriculture Organization of the United Nations—Maize and Maize Diets, A Nutritional Survey. *FAO Nutritional Studies No. 9*, Food and Agriculture Organization of the United Nations, Rome, Italy (1954).
6. Flores, M. and Reh, E. Estudios de hábitos dietéticos en poblaciones de Guatemala. I. Magdalena Milpas Altas. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 90–128, 1955.
7. Flores, M. and Reh, E. Estudios de hábitos dietéticos en poblaciones de Guatemala. II. Santo Domingo Xenacoj. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 129–148, 1955.
8. Flores, M. and Reh, E. Estudios de hábitos dietéticos en poblaciones de Guatemala. III. San Antonio Aguas Calientes y su Aldea, San Andres Ceballos. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 149–162, 1955.
9. Flores, M., and Reh, E. Estudios de hábitos dietéticos en poblaciones de Guatemala. IV. Santa María Cauqué. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 163–173, 1955.
10. Flores, M., García, B., Cruz, E., Meneses, B., Mazariegos, A. de, and Sierra, A. de. Estudios de hábitos dietéticos entre pre-escolares del área de demonstración sanitaria de Amatitlán. (In preparation.)
11. Goldsmith, G. A. Personal Communication.
12. Gross Daum, M. La niacina en el café y su importancia nutricional en Venezuela. *Arch. Venezolanos de Nutrición*, **6**, 61 (1955).
13. Hughes, E. B., and Smith, R. F. Nicotinic acid content of coffee. *J. Soc. Chem. Ind.*, **65**, 284 (1946).
14. Instituto de Nutrición de Centro América y Panamá. Recomendaciones nutricionales diarias para las poblaciones de Centro América y Panamá. Revisadas, Septiembre de 1954. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutricion de Centro América y Panamá," p. 225–226, 1955.
15. Reh, E. and Fernandez, C. Condiciones de vida y de alimentación en cuatro grupos de población de la Zona Central de Costa Rica. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 66–89, 1955.
16. Sogandares, L., Galindo, A. P. de, Mejía, H. P. Estudios dietéticos de grupos urbanos y rurales de la República de El Salvador. *Suplemento No. 1 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 27–37, 1953.
17. Sogandares, L. and Barrios, G. Estudios dietéticos en Panamá. I. La Mesa, Provincia de Veraguas. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 38–46, 1955.
18. Sogandares, L., Barrios, G. de, Corcó, E. Z. de. Estudios dietéticos en Panamá. II. Barrio El Chorrillo, Ciudad de Panamá. *Suplemento No. 2 del Boletín de la Oficina Sanitaria Panamericana*, "Publicaciones Científicas del Instituto de Nutrición de Centro América y Panamá," p. 47–53, 1955.
19. Teply, L. J., Krehl, W. A. and Elvehjem, C. A. Studies on the nicotinic acid content of coffee. *Arch. Biochem.*, **6**, 139 (1945).
20. Teply, L. J. and Prier, R. F. Nutrients in coffee. Nutritional evaluation of coffee including niacin bioassay. *J. Agr. Food Chem.*, **5**, 375 (1958).
21. The Pharmacopoeia of the United States of America, 12th ed., 1st. Sup., p. 69, 1943.

FREE AMINO ACIDS AND THIAMINE OF APPLE JUICES AND THEIR RELATION TO FERMENTATION [a, b]

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Preliminary investigations together with a review of the literature suggested that amino acids and thiamine of apple juices might play highly influential roles in their alcoholic fermentation. Also, deficiences of these soluble nitrogen compounds might be inherent causes for slow rates of fermentation and low yields of alcohol in some varieties of apple juices.

Apples contain an average of 0.01% nitrogen as compared with approximately 0.08 to 0.10% for Vinifera grapes, blackberries, currants and raspberries *(29)*. Burroughs and Pollard *(8)* reported that amino acids constituted the greater part of the nitrogen of apple juices and that asparagine was prominent in juices of high nitrogen content. Asparagine, aspartic acid, and serine were found to be the principal free amino acids present in Newton Pippin apples. Small amounts of valine, leucine, and isoleucine were detected *(18)*. Aspartic acid and glutamic acid appearing to exist free or as simple peptides or amides, were reported to be present in Baldwin apples *(12, 13)*.

The role of nitrogen in the fermentation of apple juices was studied by Barker *(2)* in 1908 who found that the addition of nitrogenous substances such as ammonium tartrate, peptone, and asparagine increased the rate of fermentation. The use of ammonium salts and/or yeast extract to facilitate fermentation has been reported by Grove *(15)*, Burroughs and Pollard *(8)*, and Clark *et al. (11)*. In general, for thiamine treatments, an increased rate of fermentation proportional to the amount of yeast growth has been reported *(7)*. Schultz *et al. (21)* showed that thiamine stimulated rate of fermentation by bakers' yeast.

According to White *(27)* brewers' yeast can synthesize needed protein, enzymes and nucleic acids from ammonia. Amides, α-amino acids, and simple peptides are good sources of nitrogen. A mixture of amino acids was found greatly superior to a single nitrogenous source for the metabolism of brewers' yeast *(3, 26)*. Polypeptides and proteins cannot be utilized owing to their great molecular size which prevents their passage into the cell and cellular proteolytic enzymes capable of splitting such large particles to assimilable fragments are not secreted by yeast *(27)*.

Castor *(10)* reported a rapid increase in yeast cells during the initial 50 hours of fermentation of grape must; a sharp decrease in amino acids of the must was noted in this same period. Amino acids of wort were also assimilated

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within 48 hours after inoculation. Amino acid assimilation represented 70% of the total nitrogen uptake *(4)*.

EXPERIMENTAL MATERIALS AND METHODS

Preparation of juices. Juices were prepared from 5 varieties of apples grown in the University of Massachusetts orchards and harvested in 1954 and 1955. Red Delicious, Rhode Island Greening, McIntosh, Red Spy and Baldwin apples were used. Apples were washed with a brush in tap water, chilled, and then dried with clean towels before being juiced. Sections of apples were juiced in a Sweden Speed Juicer [4] of aluminum, plastic, and stainless steel construction. The expressed juice was run through cheese cloth; from time to time the wet pulp was squeezed in the cloth to free the juice. The juice was stored in aluminum or stainless steel stockpots for 24 to 30 hours at 2° C. (35° F.). At the end of this time, floating sediment was skimmed off and the supernatant liquid decanted (or siphoned). The relatively clear juice was frozen in gallon jugs and stored at −18° C. (0° F.) until ready for use.

Microbiological assays for amino acids. Preliminary investigations indicated that some amino acids were present in small amounts in the juices and that a tendency toward "drifting" of the assay values occurred. Consequently, amino acids were added to the samples in order that they might be diluted to help alleviate the drift and still have values occur on a working portion of a standard curve *(28)*. A control sample of juice was assayed along with the juice containing added amino acid. Altogether a total of 30 tubes were set up for each juice in the determination of a given acid. Eleven apple juice samples were analyzed for 18 amino acids. They are listed in Table 1 together with the microorganisms and methods employed for the assay work. Stock cultures were obtained from the American Type Culture Collection. The juices were clarified prior to assay work with the aid of Celite, using 8 g. of this substance for every 200 ml. of juice. Recovery studies were conducted with isoleucine and phenylalanine by adding a known amount of one to an apple juice before the clarification procedure.

Total nitrogen. For determining total nitrogen in apple juices, a micro-Kjeldahl procedure was used. Equal parts of digestion mixture *(19)* and juice were used. The use of Dow Corning Antifoam AF Emulsion [5] was necessary to control the foaming of the material during digestion.

Thiamine. Thiamine was determined on all juice samples using the thiochrome method *(1)*.

Yeast counts of juice and inoculum. Bacto potato dextrose agar was used for pouring plates. Counts were made for yeasts and molds after a five day incubation period at 25° C. (77° F.).

Preparation of inoculum. Agar slants (Bacto dextrose broth + 1.5% agar + 1.5% added dextrose) of *S. ellipsoideus* 111A (Dept. of Bacteriology, Univ. of Mass.) were incubated for 48 hours at 25–27° C. (77–81° F.). Then a loopful of cells was transferred to 100 ml. of dextrose broth (Bacto dextrose broth + 1.5% added dextrose + 0.1% yeast extract). After 30 hours incubation at 25–27° C. (77–81° F.) the cells were centrifuged and washed with sterile distilled water. Following their transfer to a small bottle containing glass beads the cells were agitated vigorously to disperse clumps. The suspensions were made to volume (100 ml.) and an aliquot of 20 to 40 ml. diluted to 100 ml. was adjusted with sterile water to obtain a transmittance of 35% at 640 mμ. When necessary, yeast cells were activated by incubating them in dextrose broth acidified with tartaric acid (10 ml. broth + 0.1 ml. 1:10 tartaric acid solution) prior to the incubation period on agar slants.

Fermentations. Six ml. of inoculum were added to each flask containing 115 ml. of apple juice (unheated or raw) or medium. The flasks were immersed to the level of liquid therein in a constant temperature water bath, maintained at 27° C. ± 0.5° (80.6° F. ± 0.9°). A rubber stopper in each flask had one outlet that permitted escape of gas evolved and one for removal of samples. Every 36 hours 5 ml. of medium or cider was removed for alcohol determinations.

Modified juices and synthetic mixtures. Amino acids and thiamine were added

[4] Sweden Freezer Manufacturing Co., Seattle, Washington.
[5] Dow Corning Corporation, Midland, Michigan.

to selected apple juices in order to determine the role of these compounds in the formation of alcohol. In some cases vitamins and salts were also added in the event they might be critical factors lacking in the juices. In one series of experiments amino acids were added to give several levels of nitrogen, with the maximum level based on the "free amino acid nitrogen" in faster fermenting juices. Another series of additions was based on total nitrogen (by micro-Kjeldahl).

If glutamic and aspartic acids occurred as glutamine and asparagine *(16)* in the juices, then the amount of nitrogen in the amide would be twice that in the dicarboxylic acid form. Because microbiological assays indicated that the 2 acids or their amides were prominent in apple juices, a series of addition studies was undertaken with the assumption that glutamine and asparagine were to be found in juices. Generally, for all series, an effort was made to maintain the proportions of amino acids similar to their ratios in the faster fermenting juices. Synthetic media, in which different sources of nitrogen were incorporated, were prepared and fermented. The synthetic medium was based on that used by White (*27*). Analyses for alcohol were made on all samples and data on gas volumes, soluble solids and titratable acidity were obtained.

Levulose, dextrose, and sucrose were used in the preparation of a synthetic fermentation mixture. On the basis of data in Smock and Neubert *(23)* their relative proportions were 18:8:11, respectively. Approximately 11.6% total sugar was incorporated. Small amounts of malic acid were also added. Very small amounts of hydrochloric acid were employed to adjust the pH of synthetic solutions to 3.5–3.75 which zone lay between the extremes of apple juices used in the present study.

Because amino acids, vitamins, and some salts were added in solution form with resultant dilution of the juices, controls (with water in place of solutions) were set up at the same time.

Estimation of alcohol. The method of Shupe and Dubowski *(22)* for the determination of ethyl alcohol in blood and urine was used to follow alcohol formation.

Gas evolved (carbon dioxide). During fermentation studies with modified juices and synthetic mixtures gas produced was collected by displacement of saturated brine[f] from graduated cylinders. Fermentation flasks were connected to glass tubes having outlets in the inverted cylinders filled with brine. Corrections were applied for temperature, atmospheric pressure, and vapor pressure.

Titration of juices and ciders. Since sharp end points were impossible with phenolphthalein as an indicator, titrations were performed with the aid of a mixture of bromthymol blue and phenol red; the color changes sharply from blue green to purple between pH 7.4 and 8.0 *(6)*. Aliquots of sample were brought to incipient boiling before being titrated with 0.1 N sodium hydroxide.

RESULTS AND DISCUSSION

Throughout this section those juices prepared from the apple crop of 1954 will have a "−1" following the varietal designation while those from the 1955 crop will have a "−2" after the varietal name.

Amino acids. Throughout the course of assay work, the results obtained were indicative of inhibitory substances which depressed bacterial responses. This same difficulty has been encountered by others. Castor *(9)* reported that while tannins interfered with assays of grape must, other substances were concerned also. Davis *(12)* decolorized apple tissue hydrolysate to overcome inhibition of assay organisms. He suggested that inhibitors might be products of a reaction between sugars and organic acids.

Results of assays for 18 amino acids in 11 apple juices are presented in Table 2. Some of the compounds listed in Table 1 were not found in any juices; consequently they were omitted from the present table. Aspartic and glutamic acids and serine were most predominant in the juices. Results of recovery studies with isoleucine and phenylalanine indicated that the filter aid, Celite, did not retain amino acids.

[f] Electrolytes cause a "salting out" of unreactive gases in water, i.e. the solubility is decreased *(14)*.

TABLE 1

Microorganisms and references to methods for assaying eighteen amino acids

Amino acid	Microorganism	Reference
Alanine	*Leuconostoc citrovorum* [1]	Steele *et al. (24)*
Arginine	*Leuconostoc citrovorum* [1]	Steele *et al. (24)*
Aspartic acid	*Leuconostoc mesenteroides* [2]	Steele *et al. (24)*
Cystine	*Leuconostoc mesenteroides* [2]	Steele *et al. (24)*
Glutamic Acid	*Leuconostoc citrovorum*	Steele *et al. (24)*
Glycine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Histidine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Isoleucine	*Lactobacillus arabinosis* [3]	Sauberlich and Baumann *(20)*
Leucine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Lysine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Methionine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Phenylalanine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Proline	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Serine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Threonine	*Streptococcus faecalis* [4]	Henderson and Snell *(17)*
Tryptophan	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Tyrosine	*Leuconostoc mesenteroides*	Steele *et al. (24)*
Valine	*Lactobacillus arabinosis*	Sauberlich and Baumann *(20)*

[1] A.T.C.C. No. 8081.
[2] A.T.C.C. No. 8042.
[3] A.T.C.C. No. 8014.
[4] A.T.C.C. No. 8043.

Results of total nitrogen determinations made on the juices are summarized in Table 3; also calculated values for amino nitrogen and total nitrogen available from amino acids or amino acids and amides (glutamine, asparagine) are included for comparison. The information was used as a basis for adding nitrogenous materials to apple juices for fermentation studies. Greening-1 and Baldwin-2 contained considerably more total nitrogen than other juices studied; they contained a high quantity of nitrogen available from amino acids as well.

Thiamine. Data from the thiamine determinations are presented in Table 4. The quantities are small, ranging from 2.7 to 8.7γ of thiamine per 100 ml. of juice. However, the possibility that 3 to 5γ, if added to a juice, might affect the rate of fermentation was not overlooked.

Yeast counts. All juices contained less than 30 colonies per ml. Plate counts showed an average of 7,200,000 yeast per ml. of inoculum adjusted to 35% transmittance. Hence, each flask of 115 ml. of juice had a total of at least 43 million viable yeast cells added for fermentation.

Alcohol in fermenting juices. Those apple juices which exhibited the fastest rates and amounts of alcohol production were Greening-1, Baldwin-2, and Delicious-2 (Table 5). From 1.7 to 1.9% alcohol was produced in the first 36 hours of fermentation. In contrast, the slowest fermenting juices were Red Spy-1 and Red Spy-2; less than 0.5% alcohol was formed during the first 36 hours following inoculation of samples. Total alcohol after 108 hours was more than 4% in the fastest fermenting juices whereas from 0.85 to 1.36% was the maximum produced in the Spy juices.

Of interest is the fact that the juices with the most amino acids were the

TABLE 2

Free amino acids in apple juices

	Juices										
	1954					1955					
	Baldwin	Delicious	Greening	McIntosh	Red Spy	Baldwin	Delicious	Greening	McIntosh	McIntosh drops	Red Spy
	micrograms per milliliter										
Alanine	0[1]	5	24	0	0	4	0	0	0	0	0
Aspartic acid	38	138	190	17	15	238	239	92	46	37	15
Glutamic Acid	29	31	35	14	15	68	44	69	19	15	12
Histidine	0	2	4	0	0	T[2]	T	0	0	0	0
Isoleucine	T(1)[3]	10	5	0	T(2)	6	5	T	T(2)	T(2)	T
Lysine	T	T	0	0	0	0	0	0	T	0	0
Methionine	0	T	4	0	2	3	(2)	3	0	0	0
Phenylalanine	T	2	1	0	T(1)	T(1)	2	T	0	0	0
Proline	(1)	(4)	8	7	T	3	(3)	10	4	6	T
Serine	13	23	30	9	10	22	21	25	10	9	10
Threonine	T	4	4	T	0	5	5	T	2	4	0
Tryptophan	0	T	T	0	0	T	1	0	T	T	0
Valine	T	2	7	T	T	3	T	T	T	T	0
Totals											
mcg./ml.	82	221	312	47	45	353	322	199	83	73	37
mg./100 ml.	8.2	22.1	31.2	4.7	4.5	35.3	32.2	19.9	8.3	7.3	3.7

[1] None detected by the method employed.
[2] T=possible traces.
[3] ( ) indicates value on basis of "addition solution" to juice.

TABLE 3

Nitrogen in apple juices

Juice	Amino nitrogen from amino acids	Total nitrogen from amino acids	Total nitrogen from amino acids if asparagine and glutamine present	Total nitrogen
	mg. per 100 ml.			
1954				
Baldwin	0.9	0.9	1.6	7.3
Delicious	2.4	2.4	4.2	13.2
Greening	3.5	3.6	5.9	20.2
McIntosh	0.5	0.5	0.8	8.3
Red Spy	0.5	0.5	0.8	3.9
1955				
Baldwin	3.7	3.7	6.9	19.6
Delicious	3.4	3.4	6.4	13.8
Greening	2.1	2.1	3.7	7.0
McIntosh	0.9	0.9	1.6	8.7
McIntosh, Drops	0.8	0.8	1.3	5.7
Red Spy	0.4	0.4	0.7	3.8

TABLE 4

Thiamine in apple juices

Juice	Year	
	1954	1955
	γ/100 ml.	*γ/100 ml.*
Baldwin	5.9	8.7
Delicious	5.0	5.8
Greening	5.0	7.4
McIntosh	6.3	6.1
McIntosh, Drops	—	5.8
Red Spy	2.7	5.5

fastest fermenting whereas the slowest fermenting juices contained the least amount of amino acids. The magnitude of differences between juices containing large and small quantities of amino acids were not in the same proportions as between fast and slow fermenting juices. For example, McIntosh-1 fermented at approximately one-third the rate as did Greening-1 but contained only one-seventh the amount of amino acids. Burroughs' results reflected a similar situation *(7)*. In general, any of the 3 sets of nitrogen figures (Table 3) calculated from amino acid data appear to be better indicators or measures of potential alcohol production than does total nitrogen.

Alcohol in fermenting modified juices. Studies involving additions to Red Spy-1, Red Spy-2, McIntosh-1, and Greening-1 juices indicated that salts and vitamins did not constitute limiting factors for fermentation. Since thiamine was one of the vitamins in the supplement, it could not be considered a limiting factor in the low-alcohol producing juices. Fermentation was increased by adding amino acids (Table 6). Levels of nitrogen in McIntosh-1 juice were adjusted to 6.9 mg./100 ml. juice and 20 mg./100 ml. juice, using asparagine and amino acids found in casein hydrolysate. At the lower level, the juice produced about 4% alcohol after 108 hours, with about 1.5% produced in a 36-hour period (Table 6). At the higher level of nitrogen, as

TABLE 5

Per cent alcohol (wt./vol.) and rate of its production in fermenting apple juices[1, 2]

Sample	Hours after inoculum added		
	36	72	108
1954			
Baldwin	0.63	1.34	2.03
		0.71	0.69
Delicious	1.38	2.61	3.68
		1.23	1.07
Greening	1.94	3.78	4.66
		1.84	0.88
McIntosh	0.56	1.16	1.82
		0.60	0.66
Red Spy	0.44	0.90	1.36
		0.46	0.46
1955			
Baldwin	1.75	3.41	4.25
		1.66	0.84
Delicious	1.72	3.30	4.17
		1.58	0.87
Greening	1.03	2.19	3.22
		1.16	1.13
McIntosh	0.83	1.76	2.69
		0.93	0.93
McIntosh, Drops	0.74	1.58	2.40
		0.84	0.82
Red Spy	0.26	0.57	0.85
		0.31	0.28

[1] Figures represent alcohol in juice plus inoculum. Six ml. inoculum per 115 ml. of juice.
[2] For each sample the upper and lower values indicate the amount of alcohol present and the amount of alcohol produced in a 36-hour period, respectively.

much as 5% alcohol was formed after 108 hours, with more than 2% produced in a 36-hour period. Similar results were obtained with Red Spy-2 juice. The values for alcohol were comparable with either Greening-1 or Baldwin-2 diluted with water. The lower value of 6.9 mg. nitrogen was chosen from results in Table 3; it is the amount of nitrogen supplied by amino acids and glutamine and asparagine (if present) in Baldwin-2. The 20 mg. figure represented total nitrogen per 100 ml. of Baldwin-2 and Greening-1 juices. This value included nitrogen in free amino acids or their amides plus other forms not included in amino acid assays. Increasing nitrogen to almost 7 mg. per 100 ml. juice resulted in proportionally greater yield and rate of alcohol production than did 20 mg. Results of this series and others indicate that levels of nitrogen greater than 20 mg. are less important in alcohol production than are smaller quantities.

The possibility exists that some or all of the nitrogen in excess of amino acid nitrogen in Greening-1, Baldwin-2, and Delicious-2 is in a form(s) readily available to yeast. Such an occurrence might serve as an explanation of the differences in fermentation between Red Spy-1 and Red Spy-2. Their total amino acid nitrogen contents are practically the same; yet Red Spy-1 ferments more readily. Perhaps it contains more soluble or available nitrogen compounds other than amino acids than does Red Spy-2.

Synthetic mixtures containing 85 mg. of nitrogen per 100 ml. were prepared and fermented in an effort to determine how much and how fast alcohol

TABLE 6

Per cent alcohol (wt./vol.) and rate of its production in fermenting modified juices[1, 2]

Juice and additions	Hours after inoculum added		
	36	72	108
(a) Red Spy-2, water	0.20	0.43	0.67
		0.23	0.24
(b) Red Spy-2, salt, vitamin, glutamic and aspartic acid solutions; 1.5 mg. N/100 ml. juice[3]	0.44	0.99	1.52
		0.55	0.53
(c) as (b) but 3.5 mg. N/100 ml. juice	0.78	1.54	2.21
		0.76	0.67
(d) Greening-1, water	1.69	3.67	4.87
		1.98	1.20
(e) McIntosh-1, asparagine and amino acid mixture; 6.9 mg. N/100 ml. juice[4]	1.46	2.77	4.06
		1.31	1.29
(f) As (e) but 20 mg. N/100 ml. juice	2.16	3.92	4.85
		1.76	0.93

[1] Figures represent alcohol in diluted juice plus inoculum. Six ml. inoculum per 113–115 ml. juice.
[2] For each sample upper and lower values indicate alcohol present and alcohol produced in a 36-hour period, respectively.
[3] Nitrogen values represent amino nitrogen, i.e. in naturally occurring amino acids plus added amounts.
[4] Values represent amino acid nitrogen.

could be formed by the yeast employed for this problem. The maximum rate took place in casein hydrolysate medium during the first 36 hours and in a medium made with four amino acids during the second 36-hour interval. During each of these intervals 2.5% alcohol was formed. Total alcohol after 108 hours amounted to more than 5.5% in both media. Ammonium sulfate did not appear to be as good a source of nitrogen for yeast fermentation as urea and amino acids.

Juices such as Greening-1 and Baldwin-2 produced approximately 5% alcohol after 108 hours. Maximum rates (alcohol produced in a 36-hour period) of 2.0 to 2.1% were exhibited. Values for total nitrogen in the two juices were approximately 20 mg., considerably less than the 85 mg. in synthetic preparations. A need for much more than 20 mg. total nitrogen for rapid fermentation of the apple juices studied seems unnecessary. Of further interest is that the 85 mg. quantities represented highly soluble forms of nitrogen whereas only portions of the 20 mg. quantities might be considered soluble (as amino acids or amides).

Space does not permit presentation of the data from measurements of gas volumes. A slow fermenting juice such as Red Spy-1 evolved less than 400 ml. of gas whereas more than 2500 ml. were collected from fermenting synthetic mixtures in 108 hours. Sufficient data were accumulated on fermenting juices and synthetic media to conduct a statistical analysis of gas measurements in relation to alcohol production. Analyses for correlation and regression were calculated according to Snedecor *(25)*. Correlation coefficients of 0.99 were obtained for % alcohol and gas volumes; the "r's" were highly

significant. A significant regression of % alcohol on gas volume was found.

Titratable acidities of the apple juices varied from a low of 3.4 meq. per 100 ml. to 12.9 meq. No relationship between the alcohol producing capacity and the acidity of the juices was apparent. Some juices (Delicious-1-2, Spy-1) underwent small increases in total acidity while others (Greening-1-2, Baldwin-1-2, Spy-2) underwent small decreases in acidity during fermentation. Even though the other juices appeared unchanged the nature of the acids present might have been quite different from those in the unfermented material *(5)*.

SUMMARY

Results of microbiological assays for 18 amino acids have been reported; asparatic acid, glutamic acid, and serine were present in the largest quantities. Total nitrogen values were compared with total amino acid nitrogen. Using standardized inocula of *S. ellipsoideus,* fermentation studies were conducted with plain juices, modified juices, and synthetic mixtures.

Amount and rate of alcohol production appeared to be more closely related to nitrogen from amino acids than to total nitrogen. Juices containing total or amino acid nitrogen amounting to 20 mg. fermented at rates a little less than synthetic media containing 85 mg. per 100 ml. Differences in thiamine content of the various juices appeared to be unrelated to differences in fermentation.

LITERATURE CITED

1. Association of Vitamin Chemists. *Methods of Vitamin Assay.* 2nd ed. 1951. Interscience Publishers, Inc., New York. 301 pp.
2. Barker, B. T. P. The rate of fermentation of ciders and perries. *J. Agr. Sci.,* **3**, 1–21 (1908).
3. Barton-Wright, E. C. Some aspects of the nitrogen metabolism of yeast. *J. Inst. Brewing,* **57**, 415–426 (1951).
4. Barton-Wright, E. C., and Thorne, R. S. W. Utilization of amino acids during yeast growth in wort. *J. Inst. Brewing,* **55**, 383–386 (1949).
5. Beech, F. W., and Pollard, A. Acid changes in cider fermentations. I. The significance of acid changes in cider making. *Ann. Rept. Agr. Hort. Research Sta.,* (1955) Long Ashton, Bristol, 159-162.
6. Burroughs, L. F. The determination of the titratable acidity of apple juice and cider. *Ann. Rept. Agr. Hort. Research Sta.,* (1946) Long Ashton, Bristol, 127-132.
7. Burroughs, L. F. The control of fermentation in cider. II. Further observations on the influence of nitrogen and accessory growth factors on the stability of centrifuged ciders. *Ann. Rept. Agr. Hort. Research Sta.,* (1952) Long Ashton, Bristol, 110-125.
8. Burroughs, L. F., and Pollard, A. The role of nitrogen and vitamin B_1 in cider-making. *Ann. Rept. Agr. Hort. Research Sta.,* (1953) Long Ashton, Bristol, 184-188.
9. Castor, J. G. B. The free amino acids of musts and wines. I. Microbiological estimation of fourteen amino acids in California grape musts. *Food Research,* **18**, 139-145 (1953).
10. Castor, J. G. B. The free amino acids of musts and wines. II. The fate of amino acids of must during alcoholic fermentation. *Food Research,* **18**, 146-151 (1953).
11. Clark, D. S., Wallace, R. H., and David, J. J. Factors affecting the fermentation of apple juice. *Appl. Microbiol.,* **2**, 344–348 (1954).
12. Davis, S. G. The composition and nature of apple protein. (Doctor's thesis, 1948, University of Massachusetts. 70 pp.)
13. Davis, S. G., Fellers, C. R., and Esselen, W. B., Jr. Composition and nature of apple protein. *Food Research,* **14**, 417-428 (1949).

14. GETMAN, F. H., AND DANIELS, F. *Outlines of Theoretical Chemistry*. 6th ed. 1937. John Wiley and Sons, Inc., New York. 662 pp.
15. GROVE, O. The relation between the rate of fermentation and the content of nitrogenous matter in apple juice. *Ann. Rept. Agr. Hort. Research Sta.*, (1919) Long Ashton, Bristol, 20-22.
16. HAWK, P. B., OSER, B. L., AND SUMMERSON, W. H. *Practical Physiological Chemistry*. 13th ed. 1954. Blakiston Co., Inc., New York. 1439 pp.
17. HENDERSON, L. M., AND SNELL, E. E. A uniform medium for determination of amino acids with various microorganisms. *J. Biol. Chem.*, **172**, 15–29 (1948).
18. JOSLYN, M. A., AND STEPKA, W. The free amino acids of fruits. *Food Research*, **14**, 459-467 (1949).
19. PETERS, J. P., AND VAN SLYKE, D. D. Quantitative Clinical Chemistry. Vol. II, 1st ed. 1932. Williams and Wilkins Co., Baltimore. 957 pp.
20. SAUBERLICH, H. E., AND BAUMANN, C. A. The effect of dietary protein upon amino acid excretion by rats and mice. *J. Biol. Chem.* **166**, 417–428 (1946).
21. SCHULTZ, A. S., ATKIN, L., AND FREY, C. N. A fermentation test for Vitamin B_1. *J. Am. Chem. Soc.*, **59**, 948–949 (1937).
22. SHUPE, L. M., AND DUBOWSKI, K. M. Ethyl alcohol in blood and urine. A simple photometric method for its forensic determination. *Am. J. Clinical Pathology*, **22**, 901-910 (1952).
23. SMOCK, R. M., AND NEUBERT, A. M. *Apples and Apple Products*. 1st ed. 1950. Interscience Publishers, Inc., New York. 486 pp.
24. STEELE, BETTY F., SAUBERLICH, H. E., REYNOLDS, M. S., AND BAUMANN, C. A. Media for *Leuconostoc mesenteroides* P 60 and *Leuconostoc citrovorum* 8081. *J. Biol. Chem.*, **177**, 533-544 (1949).
25. SNEDECOR, G. H. *Statistical Methods*. 4th ed. 1946. Iowa State College Press, Ames, Iowa. 485 pp.
26. THORNE, R. S. W. Nitrogen metabolism of yeast. A consideration of the mode of assimilation of amino acids. *J. Inst. Brewing*, **55**, 201-222 (1949).
27. WHITE, J. *Yeast Technology*. 1st ed. 1954. John Wiley and Sons, Inc., New York. 432 pp.
28. WOOD, E. C. The theory of certain analytical procedures, with particular reference to microbiological assays. *Analyst*, **71**, 1-14 (1946).
29. YANG, H. Y. Fruit wines. Requisites for successful fermentation. *J. Agr. Food Chem.*, **1**, 331–333 (1953).

MEAT TENDERIZATION. III. HYDROLYSIS OF ACTOMYOSIN, ACTIN AND COLLAGEN BY PAPAIN[a]

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The mechanism of the tenderization of beef by papain can be effectively studied by physical *(7)*, chemical *(4)* and histological *(7, 9)* techniques using unfractionated beef and commercial papain. To supplement these studies it is desirable to have information at the molecular level using purified beef proteins as reactants and crystalline papain as the catalyst. This paper reports the results of some studies designed to supplement our previous research *(4, 7)*.

EXPERIMENTAL

Materials. The commercial proteolytic enzymes Rhozyme P-11, Protease 15 and Rhozyme A4 were obtained from the Rohm and Haas Company; commercial ficin from the Mann Research Laboratories; crystalline trypsin from General Biochemicals, Inc.; crystalline chymotrypsin from the Armour Research Division; crystalline bromelin from the Pineapple Research Institute. The proteolytic activity of all these enzymes except chymotrypsin was measured by hemoglobin assay and was reported previously *(4)*. Crystalline papain was prepared from dried papaya latex according to the procedure of Kimmel and Smith *(3)*. Purified actomyosin was obtained from rabbit muscle by the method of Weber *(10)*. Actin was prepared also from rabbit muscle by the method given by Szent-Gyorgyi *(6)*. Purified collagen was prepared from defatted freeze-dried beef tendon powder according to the method A of Baker, Lampitt and Brown *(1)*.

Methods. The concentration of the proteins and the enzymes used in this study was determined by micro-Kjeldahl analysis, assuming a nitrogen content of 16 per cent for all proteins. Enzyme hydrolysis of actomyosin, actin and collagen was carried out in similar reaction systems. Generally the reaction system consisted of 8 mg. actomyosin or other purified protein in 1.5 ml. 0.6M KCl, and was buffered with 0.2M sodium citrate at pH 6.00. For trypsin and chymotrypsin only, the substrate solution was buffered with 0.2M sodium phosphate at pH 8.04. A pH of 8 was used because of the high pH optimum of trypsin and chymotrypsin; it is not a practical condition for meat tenderization. One ml. of enzyme solution containing 0.1 mg. enzyme was added to initiate hydrolysis. The activators versene and cysteine were included in the solutions of papain, bromelin and ficin. Both enzyme and protein solutions were prewarmed in a constant water bath to the experimental temperature before mixing together. The reaction was stopped by adding 2.5 ml. of 10% trichloroacetic acid after digestion for one hour. The trichloroacetic acid precipitate was removed by filtration. The content of amino acids of the filtrate was determined by the spectrophotometric ninhydrin method of Troll and Cannan *(8)*. Alanine was arbitrarily chosen as the standard for the conversion of optical density readings into the concentrations of amino acids. Small peptides which are formed during hydrolysis and which were not precipitatable by addition of trichloroacetic acid would also contribute some color.

RESULTS AND DISCUSSION

Papain, bromelin, trypsin, ficin and Rhozyme P-11 are very active in hydrolyzing meat proteins *(4)*. The results in Table 1 show that these same enzymes readily hydrolyze actomyosin. Of the crystalline enzymes tested,

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TABLE 1

Hydrolysis of actomyosin by proteolytic enzymes at 40° C.

Enzyme at a concentration of 0.1 mg./assay		Amino acid produced in 1 hr., mole x 10^5
Crystalline	Papain	4.1
	Bromelin	2.5
	Trypsin	1.6
	Chymotrypsin	2.0
Commercial	Ficin	2.3
	Rhozyme P-11	2.9
	Protease 15	0.8
	Rhozyme A4	2.0

papain had the greatest activity. Of the 3 fungal enzymes tested, Rhozyme P-11 showed the greater activity in hydrolyzing actomyosin.

The heat stability of papain generally allows a maximum hydrolysis of proteins at 60° C. or higher. For example, the papain catalyzed hydrolysis of soybean meal *(2)* and beef *(7)* is a maximum at 60° C. The results in Figure 1 show that the papain catalyzed hydrolysis of actomyosin follows the

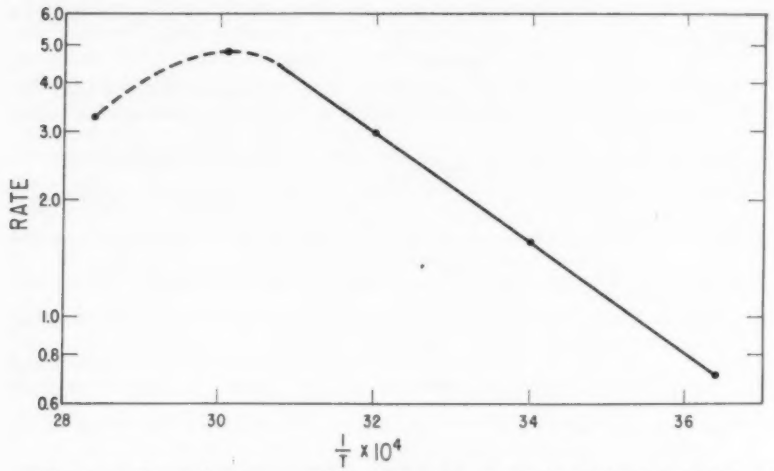

Figure 1. The Arrhenius correlation of the rate of papain catalyzed hydrolysis of actomyosin in mole x 10^5 amino acid produced per hour and the temperature.

Arrhenius relationship up to 60° C. before heat denaturation of papain shows a pronounced effect. The activation energy for this reaction is 6.5 kg. cal. per mole. This is significantly lower than papain hydrolysis of hemoglobin, 8.3 kg. cal. per mole, *(7)* and greatly lower than papain catalyzed milk-clotting, 16 kg. cal. per mole, *(7)* showing that papain hydrolysis of actomyosin is energetically the more efficient reaction.

A comparison of papain hydrolysis of actomyosin, actin and collagen is shown in Table 2. Compared to actomyosin, actin was hydrolyzed to a

limited extent, suggesting that the myosin component may be the one readily attacked by papain. As expected from previous results on papain hydrolysis of collagen in meats *(4)* and hydrolysis of collagen by various proteolytic enzymes *(5)*, crystalline papain slowly hydrolyzed purified collagen. Collectively, these results show good agreement with research on papain hydrolysis of beef proteins showing that papain is most active in hydrolyzing the muscle fiber protein, actomyosin.

TABLE 2

Hydrolysis of actomyosin, actin and collagen at 27° C.

Reactant	Amount of papain	Time	Amino acid produced
	mg.	*min.*	*mole x 10⁶*
7.9 mg. actomyosin	0.16	90	2.53
7.0 mg. actin	0.21	150	0.20
7.8 mg. collagen	0.16	90	0.24

SUMMARY

Compared to other proteolytic enzymes papain is more active in hydrolyzing actomyosin. Papain catalyzed actomyosin hydrolysis had a low activation energy of 6.5 kg. cal. per mole and proceeded faster than actin or collagen hydrolysis.

LITERATURE CITED

1. Baker, L. C., Lampitt, L. H., and Brown, K. P. Connective tissue of meat. III. Determination of collagen in tendon tissue by the hydroxyproline method. *J. Sci. Food Agri.*, **5**, 226 (1954).
2. Johnson, V. L., and Smith, A. K. Hydrolytic treatment of soybean protein with papain. *Cereal Chem.*, **25**, 77 (1948).
3. Kimmel, J. R., and Smith, E. L. Crystalline papain 1. Preparation, specificity, and activation. *J. Biol. Chem.*, **207**, 515 (1954).
4. Miyada, D. S., and Tappel, A. L. The hydrolysis of beef proteins by various proteolytic enzymes. *Food Research*, **21**, 217 (1956).
5. Sizer, I. W. The action of trypsin and other proteases on collagen. *Enzymologia*, **13**, 293 (1949).
6. Szent-Györgyi, A. *Chemistry of Muscular Contraction.* 1951. p. 148. Academic Press, New York, N. Y.
7. Tappel, A. L., Miyada, D. S., Sterling, C., and Maier, V. P. Meat tenderization II. Factors affecting the tenderization of beef by papain. *Food Research*, **21**, 375 (1956).
8. Troll, N., and Cannan, R. K. A modified photometric ninhydrin method for the analysis of amino and imino acids. *J. Biol. Chem.*, **200**, 803 (1953).
9. Wang, H., and Maynard, N. Studies on enzymatic tenderization of meat. I. Basic technique and histological observations of enzymatic action. *Food Research*, **20**, 587 (1955).
10. Weber, H. H. Muskelproteine. *Biochim. Biophys. Acta.*, **4**, 12 (1950).

THE APPLICATION OF THE BURRI METHOD FOR DETERMINING THE MICROBIAL CONTENT OF FROZEN VEGETABLES [a]

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With the introduction of microbial standards for frozen vegetables there has developed a need for a method of analysis which can be applied at the processing plant level. Most of the methods tried have had definite disadvantages. A direct microscopical examination of products is a tedious procedure and it is impossible to differentiate between living and dead cells. With some products, such as corn, due to the presence of starch, it is extremely difficult to detect bacteria. Methods to determine changes in reaction (pH) also are unreliable since bacteria may alter the pH very little or may change the pH to more acid or alkaline depending on the predominant type of organism. Much the same can be said for measurements of changes in the oxidation-reduction potential since bacteria vary in their ability to reduce the potential *(4, 5)*. Some bacteria are slow reducers while others reduce a dye, such as resazurin, rapidly. Thus, as with pH, the end result of changes in the oxidation-reduction potential of a product is largely dependent on the predominant type of organism present.

The Burri method is not new, having been used primarly for determining the microbial content of milk *(2, 3)*. Details of the method used for milk are outlined in the *Standard Methods for the Examination of Dairy Products (1)*. More recently Johns and Desmarais *(6)* have applied the method successfully for determining the microbial content of frozen egg.

To determine the relationship between counts obtained by the conventional plate method and those obtained by the Burri method approximately 100 samples of miscellaneous consumer packaged frozen vegetable products were tested. These were obtained through the Inspection Service, Fruit and Vegetable Division, Marketing Service, Department of Agriculture, and included samples of peas, beans, corn, spinach and lima beans.

EXPERIMENTAL

The Burri method is particularly suited for analysis at the plant level since results are visible to the plant operator or quality control personnel in a relatively short period of time. Very little material is required to conduct the analysis according to the Burri technique. Necessary equipment includes oval culture tubes $155 \times 27 \times 17$ mm. These oval tubes are required to give greater surface for growth of colonies than is provided by standard tubes. The inoculating loop is made of 19 B and S gauge platinum wire, with an inside diameter of 4 mm. This loop will deliver onto the slope of agar approximately 0.01 ml. of diluted sample. Sterile pint-size jars or other suitable containers are required for the product and diluent water. If further dilutions of the product are required, dilution bottles with 99 ml. of sterile water and pipettes will also be required. The

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medium used in this study was Difco plate count agar, (tryptone glucose yeast agar) with added 0.5% agar to give a more solid base.

It is important to standardize the amount of medium per tube; this results in approximately the same surface exposure of medium in each tube. In this study 10 ml. of medium were added per tube. Sloping the tubes after sterilization should be such as to give a maximum slope with minimum butt. It is also important to allow freshly prepared tubes to stand for several days in the sloped position before using. Failure to observe this precaution may result in the development of coalescing colonies due to the presence of excess moisture on the surface of the agar.

The preparation of the sample for testing consists of weighing the frozen or in some instances, the unfrozen product into sterile jars. The amount of product will depend to some extent on the anticipated microbial load. In this study either 25 g. product and 225 g. sterile water or 100 g. product and 100 g. water were used. These amounts give a dilution of 1/10 and 1/2 respectively. The diluted product is shaken by hand or by mechanical shaker for at least 5 minutes to permit adequate removal of microorganisms from the surface of the material.

Inoculation of the diluted product onto the surface of the medium is the most important step of the Burri method. It is important that the loop be bent to an angle that will facilitate surface to surface contact with the agar. The flamed, cooled loop is dipped into the diluted product and the liquid taken up by the loop is transferred to the agar slope. It is necessary to contact as much of the surface of the agar as possible by moving the loop with the inoculum up and down across the surface of the agar slope. At least 2 and preferably 3 tubes should be inoculated from each sample to permit averaging of colony counts.

In preliminary studies with the Burri method a 0.001 ml. loop was used along with the 0.01 ml. loop in an effort to obtain fewer colonies on the agar slope. However, results with the smaller loop showed greater variation than those with the larger loop when counts were compared with the plate method. Consequently, the larger loop was selected.

Following inoculation the tubes are incubated in a horizontal position at room temperature or if an incubator is available at a temperature of 32° C. (89.6° F.) for periods varying from 24 to 72 hours. Results, particularly with products with a high microbial content can frequently be obtained after 24 hours incubation. Although counting of colonies may be desirable in some instances it is usually not necessary for separating high and low count products.

The factor for the 1/10 diluted product is 1,000; for the 1/2 dilution, 200. Thus, in the former if 200 colonies develop on the agar slope the bacterial content of this product would be 200,000 per g. For the latter, a colony count of 200 would give 40,000 per g. of product. Since the standard established is 100,000 per g. *(7)* the 1/10 dilution may be desirable since the number of colonies on the agar slope would be in the "countable range." However, in practice, it has been found that the 1/2 dilution, with the greater amount of product, is probably the more accurate. It is relatively simple to determine at a glance if more than 500 colonies (100,000 per g.) are present on the Burri slope. On the other hand, if the colonies are "countable" it is sufficient to say that the count is below 100,000 per g.

RESULTS

The data presented in Figure 1 show counts obtained by the Burri and by the plate count method. With the exception of one sample (plate count 10,000, Burri count approximately 200,000) the degree of correlation between counts of the 2 methods is good. Certainly it is well within the range of accuracy anticipated with this form of analysis.

A further analysis of the data in different ranges of bacterial counts shows that in the range of 0—100,000 per g., 13 of the Burri counts (41%) were lower than the plate count, 13 were higher than the plate count and 6 (19%) were approximately the same. In the count range of 100,000 to 500,000, 28 (50%) of the Burri counts were higher than the plate counts, 14 samples (25%) were lower and an equal number were approximately the same. In products over 500,000 per g., 7 samples showed higher Burri counts, 7 were lower and 7 were approximately the same.

The average counts of products in different count ranges gave the following results. In the count range of 0—100,000 per g. the average for the plate count was 46,500; for the Burri count 56,200. In the range 100,000 to 500,000 the plate counts showed an average of 289,000; for the Burri count 361,000. The averages for products over 500,000

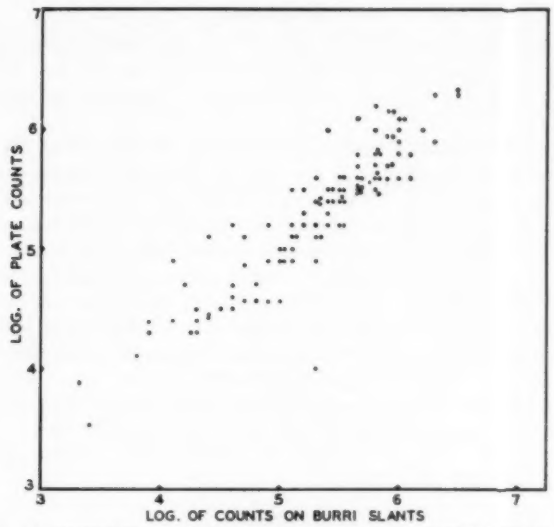

Figure 1. A comparison of bacteria counts of frozen vegetables by plate count and the Burri method.

per g. showed the plate count as 1,020,000; the Burri count 1,210,000 per gram. The overall average for the plate count was 362,000; for the Burri method 437,000. These data indicate that the Burri counts generally are somewhat higher than those obtained by the plate method.

A photograph of Burri tubes of 2 dilutions each of 6 frozen samples is shown in Figure 2. From left to right in the photograph these samples had counts of over 1,000,000,

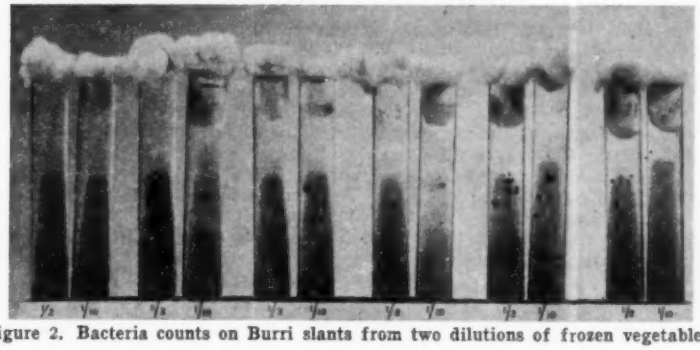

Figure 2. Bacteria counts on Burri slants from two dilutions of frozen vegetables.

200,000, 30,000, 65,000, 16,000 and 8,000 respectively. This photograph will serve to illustrate the comparative ease of interpreting results; the one sample on the extreme left shows a large number of colonies which cannot be counted. The second sample from the left shows a large number of colonies on the 1/2 dilution but colonies can readily be counted on the 1/10 dilution. Colonies on tubes from the remaining 4 samples can be readily counted.

DISCUSSION

From the data presented it is evident that the Burri method provides an inexpensive and relatively accurate procedure for determining the microbial content of frozen and unfrozen (line samples) vegetables at the processing plant level.

The Burri method may find its greatest use at processing plants for line testing of products to determine the sources of contamination. It is equally applicable for determining the efficiency of cleaning operations and of sanitizing agents. This may be accomplished using the "swab technique" along with flamed, sterilized templates with a central hole; the size of these holes may be 1 sq. cm. or any multiple thereof. The template is placed on the surface of belt, inspection table, flumes, hands of worker, etc. and a moistened sterile swab used to cover the entire area of the exposed surface. The swab is then placed in sterile water, shaken and loopfuls transferred to Burri slopes as outlined for the analysis of products.

Another obvious advantage of the Burri technique is that by varying the medium and/or the incubation temperature it is possible to detect different types of bacteria. This is particularly useful when special contamination problems are encountered.

SUMMARY

Details are given for the application of the Burri method for determining the microbial content of unfrozen (line samples) and of frozen vegetable products.

Acknowledgments

The writers are indebted to Mr. W. E. Ferguson and to Mr. A. R. Yates for providing plate counts for the products studied.

LITERATURE CITED

1. American Public Health Association. *Standard Methods for the Examination of Dairy Products.* 1933. p. 300. New York, N. Y.
2. Burri, R. Report of proceedings. World's Dairy Congress, London. 690 (1928).
3. Cunningham, A., and Andrews, A. The Burri smear-culture for the determination of the bacterial content of milk samples. *J. Dairy Research,* **5**, 29, (1933).
4. Ferguson, W. E., Yates, A. R. and Jones, A. H. Resazurin reduction by microorganisms in fresh frozen vegetables. *Food Technol.* **12**, 641 (1958).
5. Hobbs, Betty Constance. The part played by bacteria in the reduction of methylene blue in milk. *J. Dairy Research,* **10**, 35, (1939).
6. Johns, C. K. and Desmarais, J. G. The Burri slant method in the bacteriological control of frozen egg. *Food Technol.,* **5**, 36, (1951).
7. The Meat and Canned Foods Act. Processed Fruit and Vegetable Regulations. Ottawa, Canada, (1954).

QUANTITATIVE EVALUATION OF DEFROSTED *ESCHERICHIA COLI*[a]

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"Standard plate count" is an expression commonly used to describe the bacterial content of food products. It implies the detailed description of the equipment and procedures described in *Standard Methods for the Examination of Dairy Products (1)*. The sampling variation of the methods has been well ascertained and the limits of the results are generally recognized as applied to dairy products. Bacteriologists tend to use the dairy standard methods to evaluate many organisms in other kinds of samples, assuming the variables to be the same, regardless of the kind of organism or the physical and chemical nature of the original product. This assumption may or may not be valid.

Many variables which do not occur with fluid dairy products are known to affect the survival of bacteria following exposure to subfreezing temperatures. Some of these variables are the suspending medium *(8, 18, 22, 24)*, the initial cell concentration *(9, 15, 19)*, the particular species tested *(12)*, the age of the cells *(25)*, the rate of freezing or thawing *(16, 27)*, the time which elapses between thawing and plating *(17, 23)*, and the composition of the diluent *(23)* and of the plating medium *(10)*.

With certain treatments other than freezing, i.e. heat *(5, 11)*, starvation *(2, 6, 7)*, freeze drying *(26)*, or radiations *(13, 28)*, an initial lethal effect may be reversed by various reactivating agents; that is, a treated cell may either die or live depending upon the method of evaluation following the treatment. It is probable that an analogous situation exists with regard to frozen stored cells. However, determining the effect of frozen storage upon bacteria is further complicated by the fact that the number of viable organisms in a sample, while still frozen, can not be measured directly because the sample must be thawed in order to "plate it out." Thawing confounds the evaluation of any frozen storage treatment with the errors induced by the thawing process. The method giving the highest count which will most nearly approximate the "true" number of viable cells in the original material is the underlying concept of determining by plate count the effect of a treatment. Using such a criterion, this paper purports to examine the thawing and diluting variables that affect the survival of *Escherichia coli* in phosphate buffer after storage at −9° C. Thereby, we hope to show that frozen cells differ from non-frozen ones and to suggest interpretations which may explain the reasons for the observed differences. Such observations will stress underlying principles which may suggest methods for improving the analytical techniques applied to the bacteriological examination of frozen foods.

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EXPERIMENTAL METHODS

Because of their distinctive colonial morphology, two substrains of *E. coli* were selected. One was opaque (OP) and appeared to be the original parent type; the other, ground glass (GG), was a spontaneously occurring stable mutant isolated during previous studies. The terminology indicates the appearance of surface colonies when observed by means of transmitted light from below the stage in the manner described by Braun and Lewis *(3)*.

Inoculum from a stock slant was smeared onto a plate of YEVI agar (5 g. yeast extract, 5 g. peptone, 5 g. trypticase, 5 g. sodium chloride, 1 g. dextrose, 100 ml. veal infusion, 15 g. agar, and 900 ml. distilled water; pH adjusted to 7.0 with 15% KOH). After incubation for 18 to 24 hours at 37° C. the heavy growth was removed with a platinum loop to pH 7.0 M/15 phosphate buffer (3.53 g. KH_2PO_4, 5.79 g. anhydrous Na_2HPO_4, 1000 ml. double distilled water). The cells were washed 3 times in buffer by centrifugation and then resuspended in large quantities of buffer to give an optical density of 0.15 at 610 mμ (approximately 1.5×10^9 cells per ml.) with a Coleman Model 14 spectrophotometer. Aliquots of 5 ml. were dispensed into 18 × 150 mm. sterile culture tubes then covered with flat-topped aluminum caps. The tubes were "sharp" frozen at −25° C. and then stored at −9° C.

Thawing was accomplished by immersion with gentle agitation in a 37° C. water bath for 90 seconds. One-ml. aliquots were immediately diluted in the various diluting fluids using the general procedures of *Standard Methods for the Examination of Dairy Products (1)*. Triplicate pour plates or surface smeared plates were prepared from appropriate consecutive decimal dilutions into YEVI plates which were then incubated 24 to 36 hours at 37° C. before counting through a 10.5X binocular magnifier.

RESULTS

Previous storage studies had shown that with most strains of *E. coli* more than 90% of the original 10^8 cells per ml. were unrecoverable within 3 weeks, but that after 6 or 9 weeks higher counts were obtained than at 3 weeks' storage. Since cell division in a nonnutrient medium is very unlikely, how might this apparent increase be explained? Perhaps the warming and diluting process is more detrimental at one sampling time than another. Protoplasts of *E. coli* produced by the action of penicillin are stabilized by sucrose solutions. By analogy it was conceived possible that frozen cells may have their cell walls altered to make them sensitive to dilution and that they might be recovered intact if sucrose were used as a diluting fluid. Accordingly, samples were thawed and duplicate aliquots diluted in distilled water, pH 7.0, M/15 or M/2 phosphate buffer, or 10% w/v sucrose. The results are shown in Table 1.

Subsequent experiments showed that sucrose in the plating medium had no effect in increasing the counts. In addition nonfrozen samples showed similar counts with either M/15 buffer or 10% sucrose as diluent. Periodic samplings up to 33 days' storage showed that 10% sucrose usually gave higher counts than the buffer, but there was no statistically significant difference between the two diluents.

The term "osmosensitive" has been used to denote cells which may be viable while still frozen but are not recoverable unless a protective diluent is employed *(4)*.

TABLE 1

Effect of diluent on plate count of Escherichia coli after 17 weeks at −9° C.

Culture	Diluent	Viable counts x 10^4	Per cent of M/15 buffer
OP	Distilled water	107	129
	M/15 buffer	83	100
	M/2 buffer	102	123
	10% sucrose	155	187
GG	Distilled water	60	115
	M/15 buffer	52	100
	M/2 buffer	71	136
	10% sucrose	92	177

During the brief time of thawing, osmosensitive cells may be destroyed in the dilution of concentrated menstruum by water from the melting ice. Could protection be applied during the thawing process itself? Before removal from the freezer, a thawing agent of an equal amount (5 ml.) of M/15 buffer, 20% sucrose, or pH 7.0 M phosphate buffer was added to duplicate stored suspensions (in M/15 buffer). Duplicate aliquots were diluted in either M/15 buffer or 10% sucrose. After initial plating, the tubes were rapidly frozen and thawed four more times and plated again.

For stored cells Table 2 shows that (a) sucrose diluent always gave greater counts than buffer diluent, (b) sucrose added prior to thawing gave greater counts than buffer thawing agent, (c) sucrose protected many cells during subsequent freezings and thawings while buffer was very detrimental. Molar solutions of maltose or glycerol as the thawing agent produced counts similar to those of sucrose, always greater than M/15 buffer with at least 50% protection during five subsequent freezings and thawings.

Other experiments evolved a thawing agent and diluent, buffered sucrose plus magnesium sulfate, BSM, which consisted of 10^{-4} M $MgSO_4$ plus M/2 sucrose made up in M/15 phosphate buffer. The effect of the pH of the buffer and the time interval between thawing and plating were shown to be important in the following experiment. Duplicately stored samples were thawed with BSM with the pH adjusted to either 6.0, 7.0, or 8.0, then diluted in either M/15 buffer pH 7.0 or in BSM pH 7.0. They were plated immediately after defrosting and also following 10 minutes at 37° C.

It is evident from Table 3 that the two substrains differed markedly in response to the thawing agent and to time of plating, but less so to the diluent. GG increased with incubation while OP decreased; pH 7.0 was best for OP; but with GG, pH 8.0 gave greatest initial counts, while pH 6.0 yielded the most colonies after incubation.

One of the perplexities encountered in work with frozen cultures is the relative agreement of samples within any one experiment but the variability of comparative results from one experiment to another. Personal communciation with other workers in this field confirm similar difficulties. An experiment was designed to determine how much of the variation was experimental error and how much might be due to the freezing experience. Three suspensions were used, OP, GG, and a 50:50 mixture of the two. Nonaerated cells were compared with 4-hour aerated "starved," buffer-washed cells *(4)*. To ascertain sampling errors, 3 plates were made from each of 2 aliquots diluted from each of 2 duplicate tubes thawed together in each of 2 sets at different times on the same morning. There were thus 8 estimates of each storage treatment. Counts were made on replicate tubes stored 0, 3, and 6 weeks at −9° C. No added thawing agent was used and M/15 buffer was the only diluent.

Analysis of variance of the data in Table 4 showed no significant differences between each set of 8 estimates at zero time (nonfrozen). At 3 and 6 weeks, however, the difference between duplicate tubes thawed at the same time was highly significant at the 1% confidence level, but the differences between sets of 2 tubes thawed at different times was not. The coefficients of variation of nonfrozen cells were those expected from the plate count sampling procedure *(21)*, but stored samples showed increased variations. The differences between strains and between aerated and nonaerated cultures were also highly significant as is further shown in the survival curves in Figure 1.

TABLE 2

Effect of thawing agent and diluent on plate count of Escherichia coli after 7 weeks at −9° C.

Culture	Thawing agent	Viable counts x 10^9			
		After first thawing Diluted in:		After 5 freezings and thawings Diluted in:	
		M/15 buffer	10% sucrose	M/15 buffer	10% sucrose
OP	M/15 buffer	319	330	3.5	4.5
	20% sucrose	490	590	420.	440.
	M buffer	296	820	0.3	1.1
GG	M/15 buffer	30.6	35.4	1.6	1.8
	20% sucrose	266.	326.	104.	145.
	M buffer	41.4	150.	0.002	0.002

DISCUSSION

Although the use of special diluents for recovering organisms from frozen foods is not entirely new, the concept of osmosensitiveness with emphasis on the use of a solution of high osmotic strength as a diluting agent has rarely been exploited. McCleskey and Christopher *(17)* found that 50% sucrose as a diluent for *Salmonella typhosa* in unsliced sweetened strawberries gave good quantitative data while ordinary dilution water and tryptone broth were not satisfactory. Sucrose diluent for sugar syrup foods

TABLE 3

Effect of pH and time of diluting on plate count of Escherichia coli after 15 weeks at −9° C.

Culture	Thawing agent	Viable counts x 10^6 Diluted in:			
		M/15 buffer		pH 7.0 BSM	
		0 min.	10 min.	0 min.	10 min.
OP	pH 6.0 BSM[1]	125	101	150	122
	pH 7.0 BSM	237	166	212	130
	pH 8.0 BSM	151	103	228	121
GG	pH 6.0 BSM	40	138	46	148
	pH 7.0 BSM	45	128	47	115
	pH 8.0 BSM	76	97	70	76

[1] 10^{-4} M $MgSO_4$ plus M/2 sucrose made up in M/15 phosphate buffer.

TABLE 4

Effect of storage time at −9° C. on sampling variation of Escherichia coli

Culture	Weeks storage	Viable counts x 10^6 Duplicate sets								Coefficient of variation
		I				II				
		duplicate tubes A		duplicate tubes B		duplicate tubes C		duplicate tubes D		
		aliquot 1	aliquot 2	aliquot 3	aliquot 4	aliquot 5	aliquot 6	aliquot 7	aliquot 8	
OP nonaerated	0	193	231	233	186	196	196	197	219	0.084
	3	128	126	55	58	52	43	40	38	0.555
	6	76	75	30	34	45	53	45	45	0.337
OP aerated	0	170	174	194	218	171	186	172	194	0.097
	3	22	20	23	24	28	29	21	21	0.142
	6	17	17	26	27	13	16	18	18	0.258
GG nonaerated	0	121	107	118	101	124	110	105	105	0.086
	3	16	15	18	16	12	12	21	17	0.189
	6	11	10	11	12	10	12	13	12	0.088
GG aerated	0	109	128	120	110	128	117	107	97	0.096
	3	7	6	6	5	6	7	4	5	0.183
	6	7	6	8	8	5	5	7	6	0.154
Mixture of GG + OP nonaerated	0	159	149	167	150	152	184	181	166	0.080
	3	88	87	72	77	107	103	86	98	0.137
	6	85	77	47	44	64	55	21	24	0.442
Mixture of GG + OP aerated	0	151	167	149	177	203	146	145	169	0.123
	3	18	17	12	12	17	19	14	13	0.186
	6	10	10	11	12	20	16	12	12	0.271

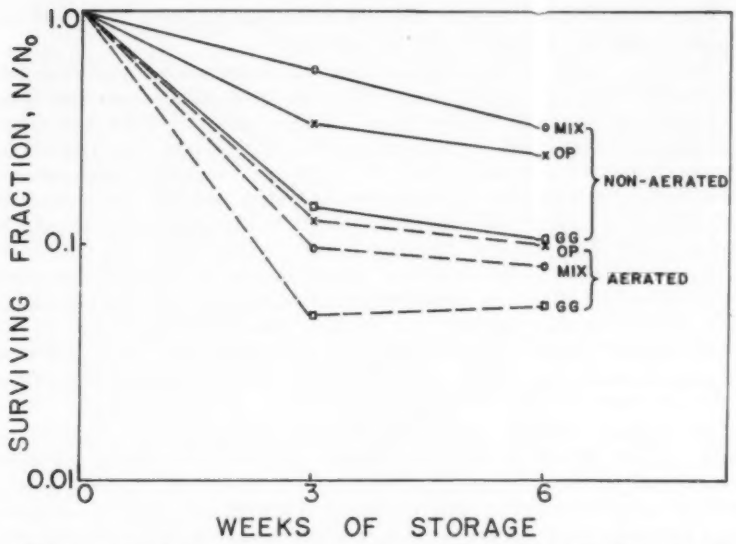

Figure 1. Survival of aerated vs. nonaerated Escherichia coli after storage at −9° C. N/N_0 is the ratio of the number of cells surviving storage divided by the initial number of nonfrozen cells.

seems logical, but the application of the osmosensitive concept to other frozen foods has not been reported. Since water freezes as pure ice, the organisms are left in a viscous concentrated inter-crystalline menstruum. It seems likely that the constituent components of a cell in such an environment differ from those of the non-stored cell. Changes in the cell wall could account for the osmosensitiveness which develops during frozen storage.

The concept of freezing death as a result of a kind of "unbalanced" growth should also be considered. In frozen cells some reactions in the metabolic chain may proceed faster than others so that upon thawing, an internal competition for cofactors or substrates may exist. There is every reason to suspect that internal contents may be lost even from viable cells in the freezing-thawing process *(2)* and that concentrated menstrua and diluents may prevent or minimize such loss, thus giving time for internal balance to be established before the cell is placed in a steady growth state again in a nutrient medium.

Accumulation of toxic or concentrated cell by-products may also contribute to cell death. Even with an osmoprotective diluent such as BSM the effect of pH is very noticeable. A rational interpretation of Table 3 is difficult because of inconsistencies in the trends. However, it is the pH effect which elucidates most clearly the differences between the two substrains of *E. coli;* thus if two closely related substrains differ it is evident that the different genera will be variously affected by any one thawing agent or diluent. Even with the same treatment two closely related substrains showed significant differences in sampling variation after storage. Certainly

the time which elapses between thawing and plating greatly influences the resultant counts of various strains of organisms.

The large coefficient of variation between frozen stored duplicates emphasizes the need for statistical control when such samples are examined. If the inherent variation between frozen tubes is very large, the comparison of treatment effects will be dependent upon replications and analysis of variance rather than a comparison of simple per cent differences between 2 treatments: 3 plates are sufficient to accurately measure the number of organisms in a given tube, but more than two separate tubes should be examined for each freezing treatment. We consider that this variability is an evidence that frozen cells have passed into a "nonnormal" sensitized state from which they may either recover or be easily destroyed by subtle manipulations in the thawing and diluting process. This idea was expressed also by Reeves and Harrison *(20)* when they said, "both death and adjustment could presumably result from the same stimulus." Conditions in the cell determine what the result is to be.

We presume that the initial evidence for freezing damage found in the osmotic effect shown by various diluents, is due to a specific metabolic process still to be ascertained. Lovelock *(14)* considers freezing damage of tissue components to be due to a denaturation of lipid-protein complexes. Since gram negative bacteria like *E. coli* are known to contain such complexes a plausible explanation for the evidence in this report is provided. The individuality of lipid-protein compounds of substrains could well account for the results reported here.

SUMMARY

Some of the principal factors involved in bacteria subjected to the frozen state have been examined. Pure substrains of *E. coli* frozen in phosphate buffer showed higher storage counts when thawing agents and diluents of increased osmotic strength were used in the Standard Plate Count technique. The term "osmosensitive" has been emphasized as pertaining to those cells which succumb to dilution in ordinary buffer or distilled water, but remain capable of producing colonies when diluted in sucrose solutions. Sampling variation of frozen cells was much greater than nonfrozen ones, thus indicating a change in stored cells and a need for multiple sampling procedures. Possible causes for the nature of the defect which contributes to the increased variation of frozen stored cells are considered.

LITERATURE CITED

1. American Public Health Association. *Standard Methods for the Examination of Dairy Products.* 10th ed., 1953. New York.
2. Bovarnick, M. R., and Allen, E. G. Reversible inactivation of the toxicity and hemolytic activity of typhus rickettsiae by starvation. *J. Bacteriol.*, **74**, 637 (1957).
3. Braun, W., and Lewis, K. H. Colony morphology of *Escherichia coli* mutants as a tool for genetic studies. *Genetics*, **35**, 97 (1950).
4. Bretz, H. W. *Frozen storage and certain life processes of Escherichia coli.* (Ph.D. thesis, 1958, Purdue University, Lafayette, Indiana).
5. Curran, H. R., and Evans, F. R. The importance of enrichment in the culture of bacterial spores previously exposed to lethal agencies. *J. Bacteriol.*, **34**, 179 (1937).

6. DAGLEY, S., AND SYKES, J. Effect of starvation upon the constitution of bacteria. *Nature*, **179**, 1249 (1957).
7. DEMAIN, A. L. Minimal media for quantitative studies with *Bacillus subtilis*. *J. Bacteriol.*, **75**, 517 (1958).
8. HARRISON, A. P. JR. Causes of death of bacteria in frozen suspensions. *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **22**, 407 (1956).
9. HARRISON, A. P. JR., ECKARDT, H. L., WEHRLE, G. W., AND PELCZAR, M. J. Survival of bacteria under various conditions of storage. *Bacteriol. Proc.* (1952) page 20.
10. HARTSELL, S. E. The longevity and behavior of pathogenic bacteria in frozen foods: the influence of plating media. *Am. J. Pub. Health*, **41**, 1072 (1951).
11. HEATER, C. D., AND VANDERZANT, W. C. Effect of the plating medium on the survival of heat-treated cells of *Pseudomonas fluorescens*. *Food Research*, **22**, 164 (1957).
12. HOWARD, D. H. The preservation of bacteria by freezing in glycerol broth. *J. Bacteriol.*, **71**, 625 (1956).
13. LATARAJET, R., AND CALDAS, L. R. Restoration induced by catalase in irradiated microrganisms. *J. Gen. Physiol.*, **35**, 455 (1952).
14. Lovelock, J. E. Denaturation of lipid-protein complexes as a cause of damage by freezing. *Proc. Roy. Soc.* (London), **B 147**, 427 (1957).
15. MAJOR, C. P., MCDOUGAL, J. D., AND HARRISON, A. P. JR. The effect of initial cell concentration upon survival of bacteria at −22 C. *J. Bacteriol.*, **69**, 244 (1955).
16. MAZUR, P., RHIAN, M. A., AND MAHLANDT, B. G. Survival of *Pasteurella tularensis* in sugar solutions after cooling and warming at sub-zero temperatures. *J. Bacteriol.*, **73**, 394 (1957).
17. MCCLESKEY, C. S., AND CHRISTOPHER, W. N. Some factors influencing survival of pathogenic bacteria in cold-pack strawberries. *Food Research*, **6**, 327 (1941).
18. MCDOUGAL, J. D. *The effect of the initial cell concentration and solute concentration upon survival of bacteria at −22C.* (M.S. thesis, 1954, Vanderbilt Univ., Nashville, Tenn.)
19. RECORD, B. R., AND TAYLOR, R. Some factors influencing the survival of *Bacterium coli* on freeze drying. *J. Gen. Microbiol.*, **9**, 475 (1953).
20. REEVES, H. C., AND HARRISON, A. P. JR. Effect of time and temperature upon survival of *Escherichia coli* in sodium chloride. *Proc. Soc. Exptl. Biol. Med.*, **95**, 278 (1957).
21. SNYDER, T. L. The relative errors of bacteriological plate counting methods. *J. Bacteriol.*, **54**, 641 (1947).
22. SQUIRES, R. W., AND HARTSELL, S. E. Survival and growth initiation of defrosted *Escherichia coli* as affected by frozen storage menstrua. *Appl. Microbiol.*, **3**, 40 (1955).
23. STRAKA, R. P., AND STOKES, J. L. Rapid destruction of bacteria in commonly used diluents and its elimination. *Appl. Microbiol.*, **5**, 21 (1957).
24. TANNER, F. W., AND WALLACE, G. I. Effect of freezing on microorganisms in various menstrua. *Proc. Soc. Exptl. Biol. Med.*, **29**, 32 (1931).
25. TOYOKAWA, K., AND HOLLANDER, D. H. Variation in sensitivity of *Escherichia coli* to freezing damage during the growth cycle. *Proc. Soc. Exptl. Biol. Med.*, **92**, 499 (1956).
26. WASSERMAN, A. E., AND HOPKINS, W. J. Studies in the recovery of viable cells of freeze-dried *Serratia marcescens*. *Appl. Microbiol.*, **5**, 295 (1957).
27. WEISER, R. S., AND HARGISS, C. O. Studies on the death of bacteria at low temperatures. II. The comparative effects of crystallization, vitromelting and devitrification on the mortality of *Escherichia coli*. *J. Bacteriol.*, **52**, 71 (1946).
28. WHITEHEAD, H. A. A factor released by irradiated cells that induces recovery of viability of ultra-violet irradiated bacteria. *Canad. J. Microbiol.*, **1**, 266 (1955).

ULTRAVIOLET EXPOSURE STUDIES ON A GAMMA RADIATION RESISTANT MICROCOCCUS ISOLATED FROM FOOD[a,b,c]

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(Manuscript received May 31, 1958)

During laboratory studies on nonsporeforming food spoilage microorganisms which are difficult to destroy by high level gamma radiation *(1)*, it was observed that such species also were resistant to ultraviolet light (UVL) irradiation. Their resistance when irradiated in phosphate buffer was greater than other nonsporeformers or the spores of *Bacillus globigii* when treated under identical conditions. The response of these microorganisms to ultraviolet light is similar in some respects to their response to gamma radiation. Mechanisms of resistance to UVL and to gamma rays may have some factors in common. It has been shown that both forms of radiation cause the formation of peroxides in the medium *(5, 7)*. If similar resistance mechanisms were indicated it was anticipated that by using UVL in place of gamma radiation perhaps some answers to problems associated with radiation sterilization could be obtained without the difficulties associated with the use of gamma rays. The ultimate objective in these experiments was to obtain information on the response of such microorganisms to irradiation under various physical and chemical environments.

This paper describes the apparatus and techniques used, together with some completed studies on a UVL and gamma resistant micrococcus.

MATERIALS AND METHODS

Cultures subjected to irradiation included: a radiation resistant micrococcus, *Micrococcus pyogenes* var. *aureus*, *Sarcina lutea* ATCC 9341, *Bacillus subtilis* morphotype *globigii* ATCC 9372 and *Escherichia coli* FDA strain 198.

Cultures to be irradiated were grown at 32° C. on a rotary shaker in 50 ml. quantities of tryptone-glucose-yeast extract (TGY) broth contained in 8 oz. prescription bottles. A 2% inoculum from a 24 hour old culture was used. Twenty-four hour cultures (except where stated) were centrifuged and resuspended in 0.05 M phosphate buffer. This resuspended cell stock on adjustment to 90% transmittance at 550 mμ with a Bausch and Lomb Spectronic-20 spectrophotometer, gave a viable plate count of $2\text{–}5 \times 10^8$ per ml. A 25 ml. aliquot of this suspension was placed in a 1×8 in. tube of UVL-transmitting glass (Vycor 7910) and irradiated in a specially constructed tank.

The irradiation tank (Figure 1) consists of a 3-chambered plastic box with a

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[a] Technical Paper No. 1129, Oregon Agricultural Experiment Station. Contributions of the Departments of Bacteriology and Food and Dairy Technology.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned No. 892 in a series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsements of the Department of Defense.

[c] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

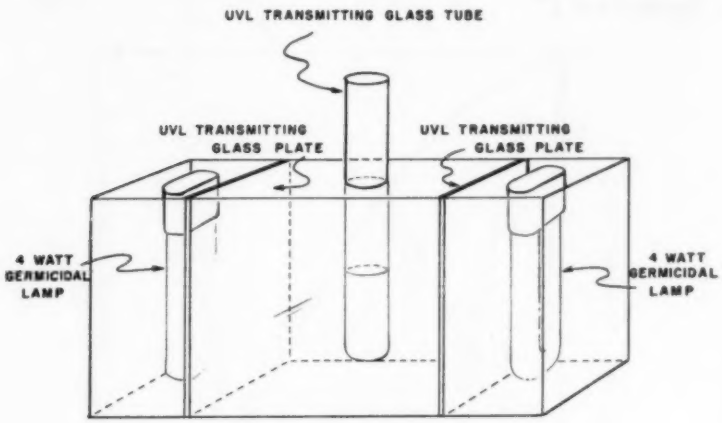
UVL TRANSMITTING GLASS TUBE
UVL TRANSMITTING GLASS PLATE
UVL TRANSMITTING GLASS PLATE
4 WATT GERMICIDAL LAMP
4 WATT GERMICIDAL LAMP

Figure 1. Ultraviolet light irradiation tank.

water-tight center section, 2 ends of which were UVL-transmitting glass plates (Vycor 7910, $6 \times 8 \times \frac{1}{8}$ in.). The 5-liter volume of the center chamber ($6 \times 8 \times 7$ in.) was filled with distilled water that could be maintained at any desired temperature during the experimental period. Four-watt germicidal lamps (G. E. G4T4/1) were suspended in each of the 2 end chambers. Germicidal intensity entering the irradiation tank was reduced by partially covering the illuminating area of the UVL bulbs, leaving only a 1.25 sq. in. opening. By reducing the UVL intensity the survival time could be extended over a 10-minute period. This facilitated sampling especially for the non-resistant bacteria which are destroyed rapidly. The UVL intensity at the center of the tank when filled with distilled water was approximately 1800 ergs/cm^2/sec. One ml. samples were taken at definite time intervals and plated on TGY agar. Results are reported as per cent survival.

Gas sparging of cell suspensions was accomplished by using Pyrex porosity EC fritted glass spargers. The suspensions were flushed for 5 to 10 minutes before irradiation to ensure complete saturation. During irradiation, a constant rate of air flow was maintained according to a rotameter. A constant rate of air flow was necessary to compare cell suspensions irradiated at different times.

Cells prepared for gamma irradiation were resuspended to approximately 5×10^7 viable cells per ml. in fresh TGY broth. Five ml. quantities were dispensed into vials and then plugged with rubber stoppers. These were then packed with sawdust in No. 2 cans and shipped frozen to the Materials Testing Reactor at Arco, Idaho, for irradiation to 1.86 megarad. at ambient canal temperature (20°–21° C.). Included as controls were extra vials for each experimental condition, treated identically, but not irradiated.

RESULTS AND DISCUSSION

The micrococcus resistant to gamma radiation is shown (Figure 2) to have a much greater resistance to germicidal UVL than *Escherichia coli* 198, *Sarcina lutea* 9341, or the spores of *Bacillus globigii*. *Sarcina lutea* was included to show that tetrad and packet formation does not account for the resistance observed in the radiation resistant tetracoccus. *Sarcina lutea* has been reported *(8)* to give sigmoidal survival curves unless corrected for tetrad and packet formation. Such corrections neither eliminate the sigmoidal characteristic, nor fully account for the resistance of this tetracoccus. The

similarities in shape of UVL and gamma radiation survival curves can be seen in Figures 2 and 3.[4]

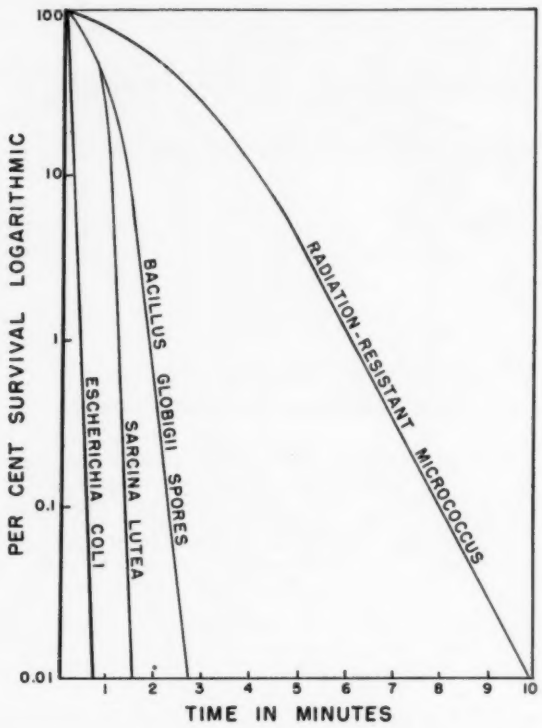

Figure 2. Survival curves comparing the UVL resistance of non-sporing bacteria and spores of a Bacillus to that of a radiation resistant micrococcus.

Culture resistance (Figure 4) appears to increase during the lag phase and reaches a maximum at approximately 2 hours at the onset of the accelerating growth phase. Following this crest, resistance drops and remains low for the rest of the growth cycle. Survival curves for the cells at various ages (Figure 5) show a delayed kill in younger cells as well as a decreased rate of inactivation throughout the 10 minute time period.

Ultraviolet light and gamma rays do not have the same effect on cells of different ages as seen by comparison of Figures 4 and 6. Gamma resistance, which is lowest in young cells, increases as the culture ages. This may indicate that different mechanisms of resistance are operative or simply that the actions of gamma and UVL causing inactivation are different. The latter possibility is supported by the differences between UVL and X-ray survival characteristics of strains of *E. coli* B and B/r *(5)* .

[4] Figure 3 is plotted from data reported earlier *(1)*.

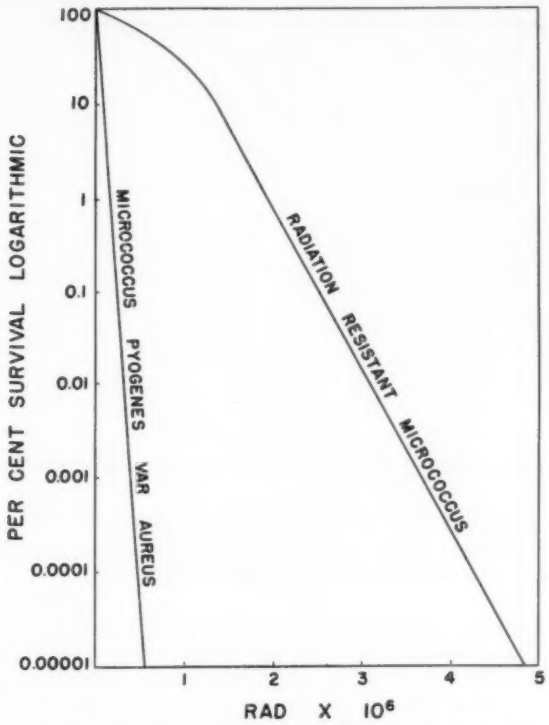

Figure 3. Gamma radiation survival curve of a radiation resistant and non-resistant micrococcus in TGY broth.

The results obtained with the radiation resistant micrococcus shows some similarity to Stapleton's X-ray data *(5)* for *E. coli*. In his results the resistance increased during the lag phase and dropped during the log phase much the same as occurred with the radiation resistant micrococcus. However, resistance of *E. coli* increased as the culture approached the maximum stationary phase. Stapleton's survival curves indicated that increased resistance during the lag phase probably could be accounted for by increased numbers of multicellular or multinuclear units in the culture. This explanation was drawn upon because of the deviation from the first order death rate during the lag phase. The radiation resistant micrococcus shows no well defined first order death rate at any age, but appears to deviate farther from logarithmic death in the lag phase (Figure 5).

Using the apparatus and techniques described, no large and consistent differences were detectable when the cells were irradiated in phosphate buffer at pH 5.3, 7.0, and 9.0. This agrees with results reported for other organisms *(2, 6, 8)*. Irradiation at 0°, 20°, 40°, 51° and 55° C. likewise showed no large differences in the rate of UVL inactivation. Workers have reported this to be true for other bacteria *(2, 6, 8)*.

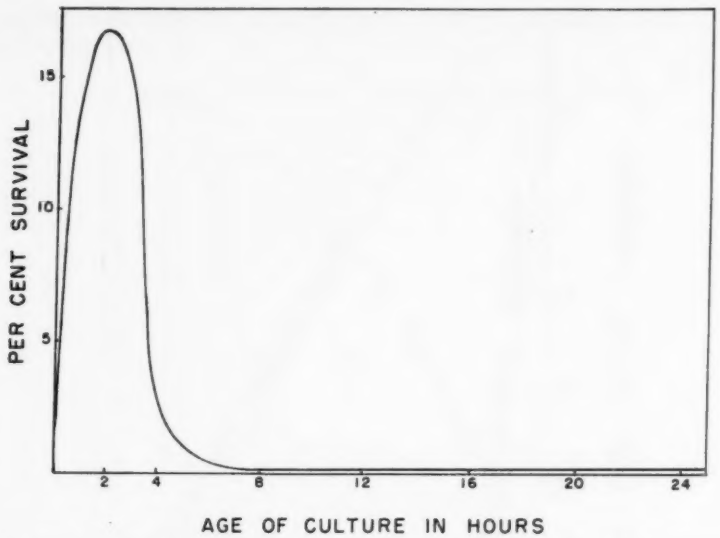

Figure 4. The effect of age on the survival of a radiation resistant micrococcus when exposed to UVL for 7 minutes in a phosphate buffer.

To determine the effect of oxygen tension on rate of kill, survivor curves were made from suspensions irradiated while being sparged with air or nitrogen. No difference in the rate of kill was observed. Oxygen has been reported to have some influnce on rates of UVL induced mutations in *Serratia marcescens (4)* but not on UVL inactivation of yeasts *(3)*. Likewise, irradiation in phosphate buffer containing 100 to 500 p.p.m. H_2O_2 resulted in no greater rate of kill than in buffer alone. The higher levels of peroxide inactivated only 20–30% of these highly catalase active cells during the 10-minute test period. Ultraviolet light has been reported to split the weak 0-0 bond of H_2O_2 to form either a pair of reactive hydroxyl radicals ($\cdot OH$) or a stable hydroxide anion ($:OH)^-$ plus a reactive hydroxide cation $(OH)^+$ *(4)*. Certain oxidations in aqueous H_2O_2 are slow or absent but are present or occur faster under UVL irradiation. The lack of a peroxide effect under UVL may indicate that the free radicals described above are not involved in UVL inactivation.

SUMMARY

An apparatus and technique for the UVL irradiation of aqueous suspensions of bacteria under controlled temperature conditions has been described. The gamma-resistant micrococcus was shown to be more resistant to UVL than other nonsporing bacteria or spores of *B. subtilis* morphotype *globigii*. Some similarities and differences in effect of age of culture on resistance to UVL and gamma radiation were pointed out. No great differences were detected in rate of kill when the radiation-resistant micrococcus was irradiated at different pH levels in phosphate buffer or at different

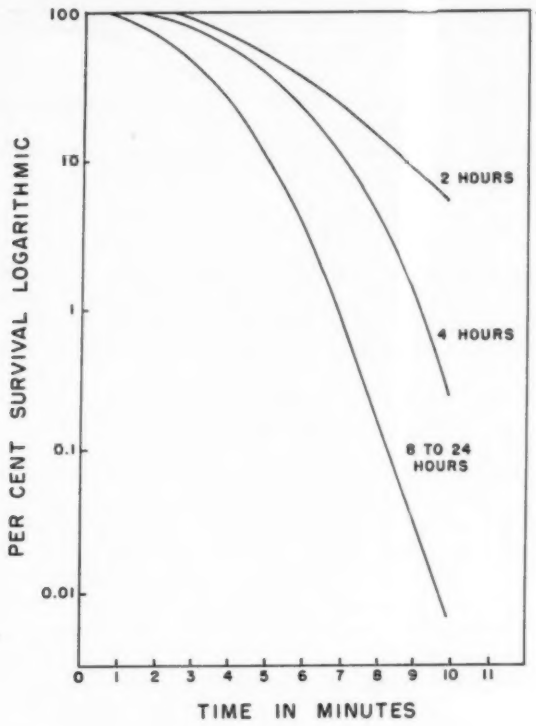

Figure 5. UVL survival curves in phosphate buffer for radiation resistant micrococcus cells at various ages.

temperatures ranging from 0° to 55° C. No differences in UVL inactivation rates were observed in the presence of oxygen, nitrogen or sublethal concentrations of H_2O_2.

LITERATURE CITED

1. Anderson, A. W., Nordan, H. C., Cain, R. F., Parrish, G., and Duggan, D. Studies on a radio-resistant micrococcus. I. Isolation morphology, cultural characteristics and resistance to gamma radiation. *Food Technol.*, 10, 575 (1956).
2. Duggar, B. M. *Biological Effects of Radiation.* Vol. 2, Chapter 34. 1936. McGraw-Hill Book Company, New York, N. Y.
3. Giese, A. C., Iverson, R. M., and Sanders, R. T. Effect of nutritional state and other conditions on ultraviolet resistance and photoreactivation in yeast. *J. Bacteriol.*, 74, 271 (1957).
4. Gilman, Henry. *Organic Chemistry.* Vol. II, 2nd ed. 1943. John Wiley and Sons, Inc. New York, N. Y.
5. Kelner, A., Bellamy, W. D., Stapleton, G. E., Zelle, M. R. Symposium on radiation effects on cells and bacteria. *Bacteriol. Rev.*, 18, 22 (1955).
6. Lea, D. E. *Actions of Radiations on Living Cells.* 1947. The Macmillan Company, New York, N. Y.

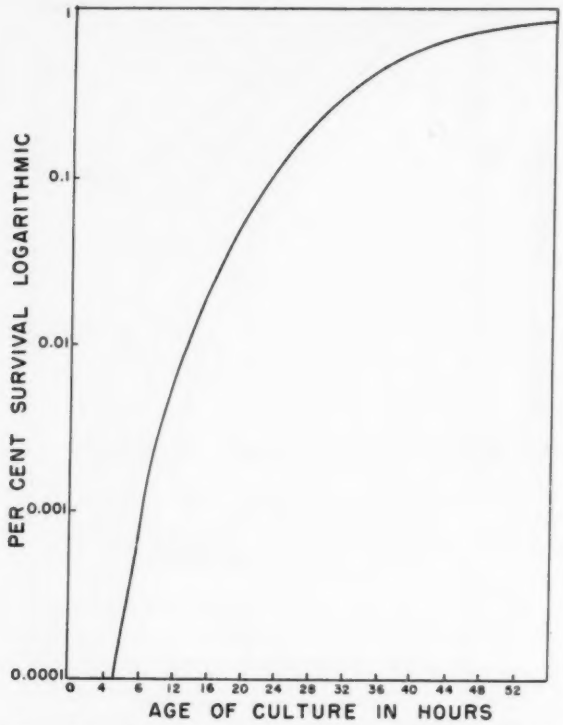

Figure 6. The effect of age on the survival of a radiation resistant micrococcus when it is exposed to 1.85 megarad in TGY broth.

7. Wyss, O., Clark, J. B., Haus, F., and Wilson, S. S. The role of peroxide in the biological effects of irradiated broth. *J. Bacteriol.*, **56**, 51 (1948).
8. Hollaender, A. *Radiation Biology.* Vol. 2, Chapter 10. 1954. McGraw-Hill Book Company, New York, N. Y.

STUDIES IN THE COLD STERILIZATION OF LIQUID FOODS USING MERCURY RESONANCE RADIATION. II. APPLE JUICE[a, b]

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(Manuscript received May 31, 1958)

Application of mercury resonance radiation to the processing of raw milk has been described in a previous report from this laboratory *(1)*. Since the results indicated that raw whole milk could be sterilized by this technique, it was considered desirable to investigate its application to the processing of other liquid foods.

Apple juice, or sweet cider, was selected as the liquid food to be studied. The clarity, light color, and low solids of apple juice allow good penetration of the readily-dispersed ultra violet rays. The low protein content and absence of fats present a minimum of photo-reactive solute constituents.

Large quantities of unpasteurized apple juice are marketed in the fall and early winter months through direct farmer to consumer sales. Since this product deteriorates rapidly as a result of microbial activity, considerable effort has been directed toward the development of methods of preservation. Filtration and heat pasteurization have been commonly used to preserve apple juice *(13, 14)*. Sodium benzoate has been used as a chemical preservative for many years *(6, 7)*. Since an undesirable flavor is often imparted to the juice by this compound *(5)*, more recent investigations have been directed towards the use of other chemical preservatives.

Salunkhe *(11)* found that sorbic acid effectively prevented the fermentation of unpasteurized apple juice by yeasts. Ferguson and Powrie *(7)* reported that 0.035% sorbic acid preserved apple juice for 4 to 10 days at 77° F. A combination of sorbic and ascorbic acid extended this period to 14 days. Weaver *et al. (15)* found that sodium sorbate preserved apple cider for 7 days at 70°–75° F., and for over 105 days at 50° F. According to this report, no objectionable off-flavors were imparted to the juice. Recently, Asselbergs *et al. (2)* reported that irradiation of apple juice with a 621,000 rep dose of gamma radiation in the presence of 0.05% sodium sorbate and 75 mg./100 ml. ascorbic acid resulted in preservation at room temperature for 13 days. However, the flavor, viscosity and color of the juice were seriously impaired by this treatment.

The objective of the present study was to determine whether or not a stable product having a satisfactory flavor could be obtained by treatment of apple juice with mercury resonance radiation.

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[a] The data presented in this paper were taken from a thesis presented by the senior author to the Graduate School of Illinois Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science in Food Engineering.

[b] Presented before the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

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[d] Present address: International Minerals & Chemical Corporation, Skokie, Illinois.

MATERIALS AND METHODS

The source of ultraviolet radiation for this experiment was a "Biosteritron" mercury vapor-argon in quartz resonance discharge tube.[e] Ninety per cent of the power output of this lamp in the ultraviolet region is emitted at a wave length of 2537Å., and 9% at 1849Å. The product was irradiated in continuous flow through a specially designed fused quartz cell,[f] which maintained a laminar film 0.008 inch in thickness. Primary radiation of approximately four "ultraviolet watts" was directed upon both sides of the film from a distance of one-eighth inch through a rapidly moving stream of nitrogen. The apple juice was deaerated prior to irradiation, and the entire fluid system maintained under nitrogen atmosphere during treatment. The maximum exposure of the juice to irradiation was 0.25 seconds per milliliter.

Raw apple juice was obtained by random purchase at local farm cider mills. The juice was sold as expressed from a rack-and-cloth press; no clarifying or preserving additives were used. The fruit used were culls and windfalls of random varieties.

Total microbial counts were made on a medium of the following composition: glucose (anhydrous), 4 g.; tryptone (Difco), 10 g.; yeast extract (Difco), 3 g.; KH_2PO_4, 0.9 g.; agar, 17 g.; and water to 1 L. The pH of this medium was 5.8. The plates were inoculated for 72 hours at 32° C. Malt agar (Difco) adjusted to pH 4 was used for yeast counts; coliform organisms were enumerated on Violet Red Bile Agar (Difco). In both instances, the plates were incubated at 30° C. for 48 hours.

Ascorbic acid was determined by the 2,6-dichlorophenol indophenol photometric method *(3)*, thiamin by the thiochrome method *(3)*, and riboflavin by the fluorometric method *(3)*.

Sensory evaluation was made by a panel of 33 members. A triangle test was used to determine the acuity of the judges. A 9-point hedonic scale comparison was made between the irradiated juice and 4 commercially marketed types of apple juice; unprocessed, pasteurized (canned), reconstituted 4× frozen cencentrate, and reconstituted 70° concentrate.

RESULTS AND DISCUSSION

Preliminary studies were conducted to determine the approximate lethal exposure required under the experimental conditions employed. The evaluation was made by comparing the initial and final total plate counts from single timed cycles through the irradiation apparatus. Random samples of commercial canned juice were used. Higher initial microbial counts were obtained by allowing an opened container to incubate at room temperature. The results showed that approximately 0.25 seconds exposure per milliliter under these conditions would effect a 90% reduction of the number of viable organisms in the apple juice.

The rates of microbial destruction were determined in the two types of apple juice: the fresh, unprocessed product, and a mixture of one-third fresh juice and two-thirds canned juice. Samples were exposed to a cumulative dosage of one minute or more per milliliter in 4 to 6 cycles of various durations. Total plate counts were made initially and after each cycle. The number of survivors was correlated with cumulative exposure time by the application of the method of least squares. Linear regression lines were calculated on an exponential scale. The correlated survivor curves for the raw and mixed juices respectively are shown in Figures 1 and 2. The average slope of each group represents an exposure of 0.38 seconds and 0.21 seconds per milliliter to effect decimal reduction[g] in the raw and mixed juices, respectively. Total

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[e] Hanovia Chemical & Manufacturing Co., Newark, New Jersey.

[f] Nieder Fused Quartz Co., Philadelphia, Pennsylvania.

[g] Reduction to one-tenth the number of viable organisms, or one unit on a logarithmic scale *(4)*.

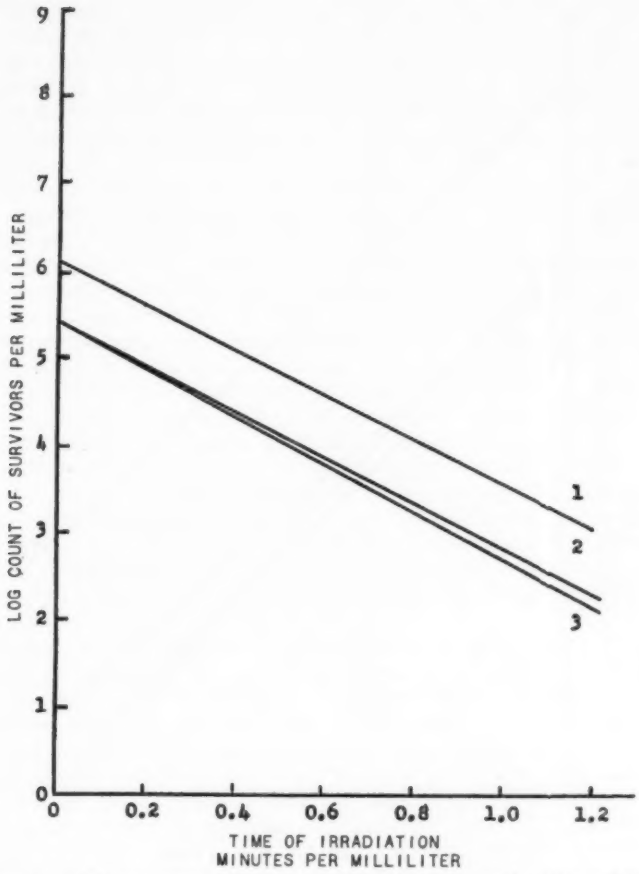

Figure 1. Survivor curves for microorganisms in fresh apple juice subjected to ultraviolet irradiation.

reductions as high as 99.9997% (5.5 units of decimal reduction) were attained by this technique.

All of the culture plates examined in these series of experimental runs revealed a mixture of yeasts and bacteria. The percentage of each appeared to remain relatively constant, although no differential counts were made. Only occasional molds appeared on the culture plates. Coliform organisms were enumerated several times during experimental runs. Table 1 illustrates the relative numbers of this group of organisms.

The survivor curve of a pure culture of microorganisms under ultra violet irradiation is normally a simple exponential expression *(10)*. The mathematical summation of a number of such curves, representing a heter-

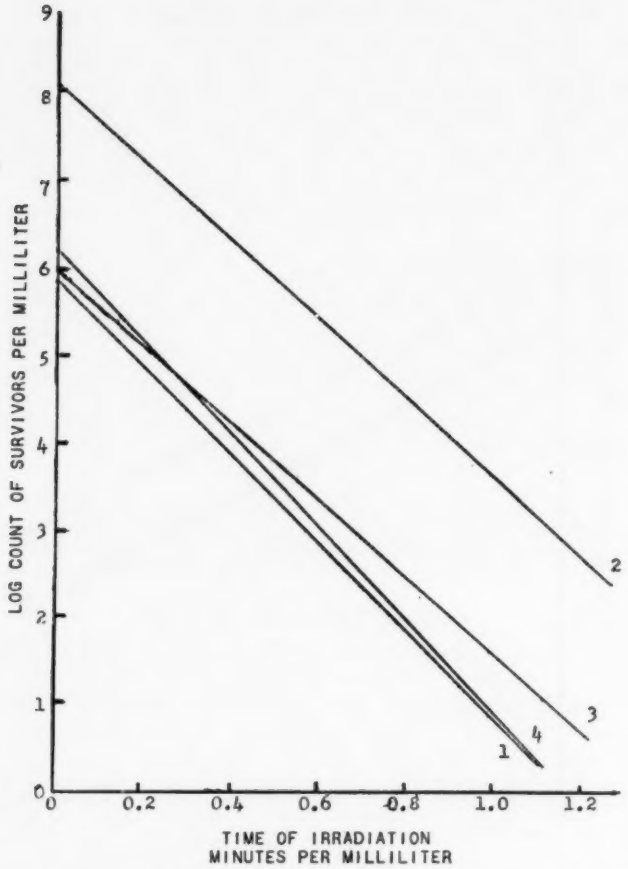

Figure 2. Survivor curves for microorganisms in a mixed apple juice subjected to ultraviolet irradiation.

TABLE 1

The effect of ultraviolet irradiation on coliform organisms in apple juice

	Coliform count/ml.	Total count/ml.
Fresh juice	20,000	1,100,000
After exposure of 0.2 sec/ml	5,200	320,000
After exposure of 0.5 sec/ml	0	7,300

ogenous mixture of organisms, would result in a complex relationship. However, the correlation coefficients obtained for the "least squares" lines presented in Figures 1 and 2 were 0.90 or better in all cases, indicating an excellent conformation to a simple exponential expression *(9)*.

The difference in death rates of the organisms between the raw and mixed juices could be attributed to 3 factors: (a) the clarity of the juice, (b) the microbial flora present, or (c) the chemical composition. The colloidal particles present in the raw juice would produce a scattering effect on light radiation not found in the clarified commercial product. It would not be expected that the organisms initially present in the commercially processed juice would be radically different from those in the apple juice obtained locally. The heat treatment, however, may have had some effect upon resistance to ultra violet light. Certain chemical constituents to the juice (e.g., vitamins, pigments) are also affected by commercial processing. Compounds of this type have been shown to exert an effect upon microbial resistance to ultraviolet light.

Since complete sterility was not attained in the experimental runs, a storage test was conducted to observe the characteristics of growth of the surviving organisms. Raw and irradiated juice of the same lot were held in partially-filled, loosely-capped bottles at room temperature (20° – 25° C.) for 9 days. Total counts and yeast counts were made at 0, 2, 4, 7 and 9 days after irradiation. The resulting growth curves are shown in Figure 3. The organisms surviving irradiation exhibited normal colony formation. The comparable forms of the growth curves in Figure 3 show no evidence of reactivation or residual effect under these storage conditions. The immediate active growth of acid-forming bacteria may be explained by the considerable yeast fermentation that had occurred in the product before irradiation. The resulting fermentation products, coupled with the lysis of the inactivated cells, would greatly enhance the nutritive value of the medium as a bacterial substrate.

In view of the recent use of sorbic acid as a preservative, it appeared desirable to investigate the combined effects of ultraviolet irradiation and this compound on the microorganisms in apple juice. A sample of commercial pasteurized juice was fortified with 0.05% sorbic acid, and immediately irradiated. The resulting survivor curve is shown in Figure 4. The same rate of destruction was obtained as without added sorbic acid, 0.25 seconds per milliliter to effect decimal reduction. Samples of this product before and after irradiation were held in partially-filled, loosely-capped bottles at room temperature (20° – 25° C.) for 8 days. The resulting microbial counts, shown in Table 2, indicated that the preservative effect of sorbic acid was not affected by irradiation, and that prevention of yeast fermentation by irradiation increased the storage life of the product.

TABLE 2

The effect of ultraviolet irradiation on apple juice preserved with 0.05% sodium sorbate

	Total count per milliliter	
	Raw juice	Irradiated juice
Before irradiation	20,000	20,000
After irradiation	—	19
After 8 days storage	33,000,000	280,000

An evaluation was made of the destruction of ascorbic acid, thiamin and riboflavin under the irradiation conditions employed. The results of the analyses for these vitamins before and after irradiation of two different unprocessed juices are presented in Table 3.

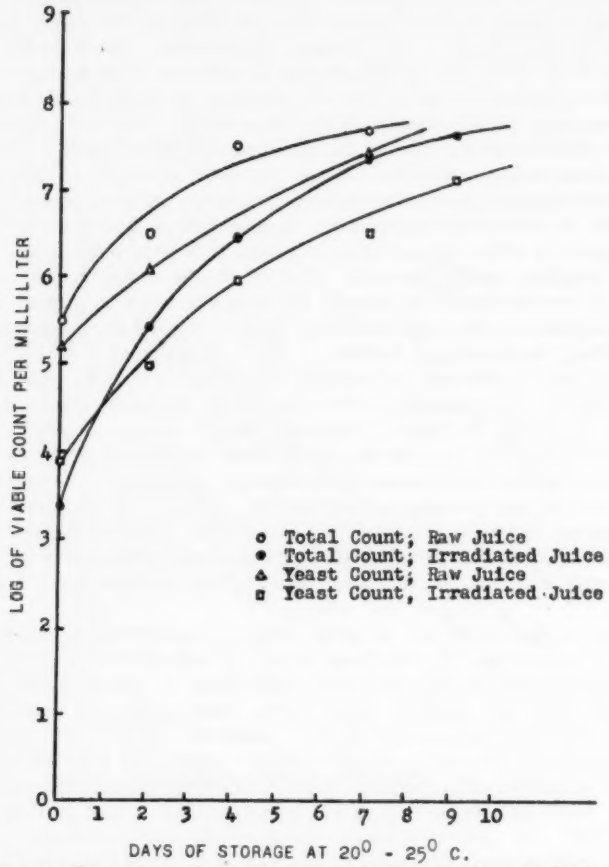
LOG OF VIABLE COUNT PER MILLILITER
DAYS OF STORAGE AT 20° - 25° C.
Total Count; Raw Juice
Total Count; Irradiated Juice
Yeast Count; Raw Juice
Yeast Count, Irradiated Juice

Figure 3. Comparison of growth curves of microorganisms in fresh apple juice with growth curves of surviving microorganisms in irradiated apple juice.

TABLE 3

The effect of ultraviolet irradiation on the ascorbic acid, thiamin, and riboflavin contents of apple juice

Vitamin	Potency, mcg./ml.	
	Fresh juice	Irradiated juice
Ascorbic acid	19.2	13.7
	28.0	19.2
Thiamin	1.13	0.81
	1.48	1.15
Riboflavin	0.16	0.15
	0.10	0.09

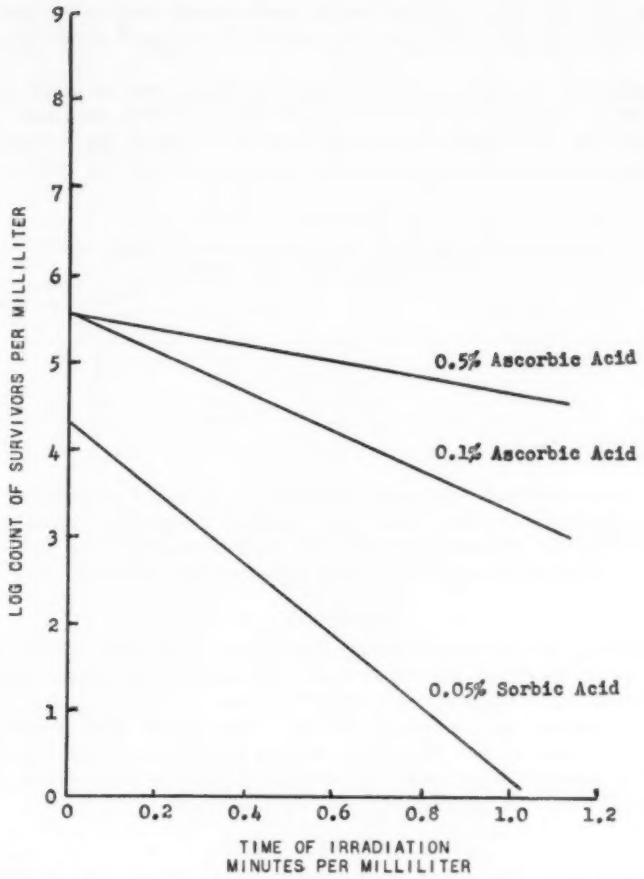

Figure 4. Survivor curves for microorganisms in apple juice containing ascorbic acid or sorbic acid.

The appreciable destruction of ascorbic acid is due to its high absorption at wave lengths near 2537Å. Recently it was reported *(8)* that ascorbic acid exerts a protective effect against destruction of microbial spores by ultra violet light in saline solution. Concentrations of 0.5% and 1.0% were reported to exert complete protection; 0.1% and 0.01%, decreased protection, and 0.001%, no effect. Two comparison irradiations were made to observe any protective effect in a natural food product. Pasteurized apple juice, inoculated with viable cultures of *Lactobacilli, Bacillus subtilis,* and *Saccharomyces cerevisiae,* was fortified with ascorbic acid to the 0.1% and 0.5% levels. The survivor curves during the irradiation of these two fortified juices are shown in Figure 4. The rate of destruction may be compared with that of the sorbic acid fortified juice, which is the same as that for unfortified juices.

Although the protective effect of ascorbic acid is confirmed by the data, the effective levels are far higher than the natural ascorbic acid content of apple juice.

A difference in flavor resulting from irradiation was observed by the taste panel in triangle tests which was significant at the 95% confidence level. The results of the hedonic scale comparison of irradiated apple juice with four commercially marketed types of this product are shown in Table 4. The

TABLE 4

Comparative evaluation of fresh commercially processed, and ultraviolet irradiated apple juices

Type of juice	Average score	Number of responses	
		Like	Dislike
Canned	6.25	25	7
Fresh	5.06	15	13
Frozen 4X concentrate	4.11	10	18
Irradiated	3.91	10	20
70 concentrate	3.09	3	28

products were rated on a nine-point descriptive scale, with 4 possible responses of varying degree in both "like" and "dislike" categories. These results indicated that the irradiated product was liked by one-third of the tasters. Comments of the panel members fell into two categories: *burnt,* or *over-sweet.*

SUMMARY

Satisfactory preservation of fresh, unprocessed apple juice can be accomplished by the action of ultraviolet light from mercury resonance radiation. Addition of sorbic acid did not affect the rate of microbial destruction; addition of ascorbic acid did reduce the rate. The ascorbic acid, thiamine and riboflavin content of the juice was reduced by ultraviolet irradiation. A noticeable flavor change resulted from dosages sufficient for preservation; however, the resultant product was definitely palatable.

LITERATURE CITED

1. Albrecht, J. J., Gunning, H. D., and Parker, M. E. Studies in the cold sterilization of liquid foods using mercury resonance radiation. I. Milk. *Food Research,* 20, 424–442 (1955).
2. Asselbergs, E. A., Ferguson, W. E., and MacQueen, E. F. Effects of sodium sorbate and ascorbic acid on the attempted gamma radiation pasteurization of apple juice. *Food Technol.,* 12, 156-15 (1958).
3. Association of Vitamin Chemists. *Methods of Vitamin Assay.* 1951. Interscience Publishers, New York, N. Y.
4. Ball, C. O., and Olson, F. C. W. *Sterilization in Food Technology,* 1957. McGraw-Hill Book Co., New York, N. Y.
5. Fabian, F. W., and Marshall, R. E. *How to Make, Clarify and Preserve Cider.* Mich. Sta. Agr. Expt. Sta. Circ. *Bull* 98 (1935).
6. Fellers, C. R. Sodium benzoate and benzoic acid, preservatives for cider and other fruit juices. *Fruit Prod. J.,* 9, 113–116 (1929).
7. Ferguson, W. E., and Powrie, W. D. Studies on the preservation of fresh apple juice with sorbic acid. *Appl. Microbiol.,* 5, 41–43 (1957).
8. Fuld, G. J., Proctor, B. E., and Goldblith, S. A. Protection of bacterial spores from the effects of ultraviolet radiation by sodium ascorbate. *Bacteriol. Proc.,* 33–34 (1958).

9. HOEL, P. G. *Introduction to Mathematical Statistics.* 1954. J. Wiley & Sons, New York, N. Y.
10. HOLLANDER, A. *Radiation Biology.* Vol. II. 1955. McGraw-Hill Book Co., New York, N. Y.
11. RICE, F. E., AND MARKLEY, A. L. *Cider and its Preservation.* N. Y. Sta. Agr. Expt. Sta. *Bull.* **44** (1921).
12. SALUNKHE, D. K. Sorbic acid as a preservative for apple juice. *Food Technol.*, **9**, 590 (1955).
13. SMOCK, R. M., AND NEUBERT, A. M. *Apples and Apple Products.* 1950. Interscience Publishers, New York, N. Y.
14. TRESSLER, D. K., AND JOSLYN, M. A. *The Technology and Chemistry of Fruit and Vegetable Juice Production.* 1954. Avi Publishing Co., Westport, Connecticut.
15. WEAVER, E. A., ROBINSON, J. F., AND HILLS, C. H. Preservation of apple cider wtih sodium sorbate. *Food Technol.*, **11**, 667–669 (1957).

CARBONYLS IN OXIDIZING FAT. II. THE IDENTITY AND AMOUNTS OF STEAM VOLATILE MONOCARBONYLS IN A RANCID FREEZER-STORED PORK FAT

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A considerable amount of reasearch on the carbonyl compounds of oxidized fat has been done by investigators of autoxidative mechanisms and reversion of vegetable oils. This work has been thoroughly reviewed by Morris *(11)* and Lea *(10)*. Conventional methods were used in the investigations and results tended to be qualitatively and quantitatively incomplete. Swift, O'Connor, Brown, and Dollear *(14)* in 1949 isolated hexanal, 2-octenal, and 2,4-decadienal from the steam distillate of autoxidized cottonseed oil. The monocarbonyl classes represented by these compounds have since proven to be the major ones present in autoxidized fat.

In recent years the use of 2,4-dinitrophenylhydrazone[a] derivatives, and the application of improved chromatographic methods has given a more complete indication of the monocarbonyls present in a product. Forss, Dunstone, and Stark *(2, 3)* in 1955, using adsorption chromatography, isolated acetone, n-saturated aldehydes C_2 and C_6, alk-2-enals C_3 and C_5–C_{11}, and 2,4-dienals C_5–C_{11} in oxidized skim milk. Recently, Pippen, Nonaka, Jones, and Stitt *(13)* identified acetone, methyl ethyl ketone, n-saturated aldehydes C_2–C_6 and C_8 and C_9, alk-2-enals C_5–C_7 and C_{10} and C_{11}, alk-2,4-dienal C_7, and diacetyl in 2,4-DNPH derivatives obtained by passing air through a simmering mixture of chicken muscle tissue and water.

The methods employed by Forss, Dunstone, and Stark *(2, 3)* and Pippen, Nonaka, Jones, and Stitt *(13)*, although having certain advantages, require relatively large quantities of 2,4-DNPH derivatives, and are laborious and time-consuming. Moreover, as shown by Forss and Dunstone *(4)* decomposition and rearrangement of the 2,4-DNPH's may occur during lengthy exposure to the adsorbents used, silicic acid and silica gel. Gaddis and Ellis *(6)* and Ellis, Gaddis, and Currie *(1)* have reported rapid paper chromatographic methods of separating monocarbonyl 2,4-DNPH's into classes and the classes into individual compounds. These micro-methods have been applied by Gaddis, Ellis, and Currie *(7)* in a preliminary study of changes taking place in the proportions of classes of the volatile monocarbonyls of freezer-stored pork tissue fat. Gaddis and Ellis *(5)* identified hexanal, propanal, ethanal, methanal, acetone, and methyl ethyl ketone in the saturated classes of a very rancid pork fat. The purpose of this paper is to report the kinds and relative amounts of steam volatile monocarbonyls in a cooked and an uncooked pork tissue fat in the early stages of rancidity.

[a] Hereinafter referred to as 2,4-DNPH.

EXPERIMENTAL

Solvents, reagents, materials, and equipment. These items were the same as were used in similar operations described in earlier papers *(1,5,6,7)*.

Materials and methods. The tissue fat sample chosen was an uncured bacon side stored at 15° F. for 15 months. The fatty tissue was ground and rendered under mild conditions *(5,7)*. Determinations were made on 10.0 g. samples of the unheated and heated fat. The fat was heated at 165° C. for 15 minutes to approximate cooking. Steam distillation, collection of the carbonyls as 2,4-DNPH's, extraction of the derivatives, and separation into monocarbonyl and dicarbonyl fractions was done as described in previous papers *(5,7)*. The total monocarbonyls were estimated by their absorbance at the wavelength of maximum absorption, and resolved into classes by the paper chromatographic method reported in *(6)* as applied by Gaddis *et al. (7)*. The separated classes were extracted from the paper, and proportions were determined in the manner described by the latter authors *(7)*. Paper chromatographic separation of the classes into individual compounds was done by the method of Ellis *et al. (1)* and Gaddis and Ellis *(6)*. The individual compounds were extracted from the paper and identified by comparison of R_F values with those of authentic compounds, non-separation or difference in R_F when mixed with authentic DNPH's, maxima in carbon tetrachloride, fading rate in alcoholic alkali of the major maxima, and the presence, dissappearance, or absence of secondary maxima around 520–535 mμ *(1,8)*. Authentic C_8–C_{10} alk-2-enal and C_9–C_{12} alk-2,4-dienal derivatives were furnished by Dr. E. L. Pippen, of our Western Utilization Research and Development Laboratory. Other 2,4-DNPH's were prepared as previously described *(1,6)*.

RESULTS AND DISCUSSION

The freezer-stored bacon side used in this study, when ground and mixed, had a peroxide value of 17. Samples from it had been tested by the method of Moser *et al. (12)* at monthly intervals up to 11 months of storage, at which time it was judged still in the good range in aroma and acceptable range in flavor. When this study was made after 15 months of storage, the flavor of the uncooked rendered fat was in the objectionable range, and the cooked in the unpleasant category. The cooked sample had a much stronger and somewhat fishy flavor.

The absorbance values in 100 ml. of carbon tetrachloride of the monocarbonyl and dicarbonyl fractions from the unheated sample were 0.87/346–9 mμ and 0.19/375–85 mμ. Values for similar fractions from the heated sample were 1.41/361–5 mμ and 0.27/349–52 mμ. These shifts in the maxima of heated mono- and dicarbonyls have been noted and discussed by Gaddis, Ellis, and Currie *(7)*.

Table 1 shows the results obtained in the separation of the monocarbonyls into classes. These values result from the combined spots from three paper strips read in 3.00 ml. of carbon tetrachloride. Amounts of the monocarbonyl solutions (100 ml.) spotted on each strip were 1.724 ml. for the unheated and 1.063 ml. for the heated. These amounts were calculated from the formula,

$$\text{x ml.} = \frac{1.5}{\text{Absorbance}}$$

(7). The total amount of material spotted for each monocarbonyl fraction should have an absorbance value of 1.499 in 3.00 ml. of carbon tetrachloride. However, in the unheated, recovery at 346 mμ was 0.953, and in the heated at 361 mμ, it was 1.229. This appears to indicate recoveries of 63.57% for the unheated and 81.99% for the heated. It has been noted *(1,6)* that losses are due to failure of some material to move from the origin, and incomplete

TABLE 1

Separation by paper chromatography of monocarbonyls into classes [1]

λmμ	Unheated fat absorbance values			Heated fat absorbance values		
	Spot			Spot		
	1	2	3	1	2	3
320	0.398	0.102		0.237	0.155	
330	0.544	0.128		0.330	0.225	
340	0.660	0.162		0.402	0.312	
343	0.670	0.170		0.414	0.336	
346	0.680 [2]	0.180	0.093	0.420	0.358	0.305
349	0.674	0.184	0.097	0.420	0.380	0.335
352	0.655	0.188	0.102	0.410	0.400	0.365
355		0.188	0.106	0.392	0.415	0.400
358		0.188	0.114	0.370	0.425	0.430
361		0.184	0.117	0.335	0.430	0.464
365		0.177	0.120		0.430	0.490
370			0.125		0.415	0.525
375			0.125		0.390	0.550
380			0.121			0.565
385			0.117			0.570
390						0.554

[1] Combined spots from 3 paper strips in 3.00 ml. CCl_4.
[2] Figures enclosed in rectangles denote maximum absorption.

extraction from the paper. Recovery has been consistently observed to be better, the higher the concentration of carbonyls. This may be due to the presence of non-carbonyl impurities which would have a greater influence at higher volumes of material spotted. Based on the absorbance at the maxima *(7)*, the data indicated 68.0% n-alkanal class, 19.0% mixture of classes, and 13.0% alk-2,4-dienal class in the unheated. In the heated, proportions were 29.6% n-alkanal, 30.3% alk-2-enal, and 40.0% alk-2,4-dienal. The group in the unheated monocarbonyls, which is composed of a mixture of classes, has been referred to in early work *(5, 7)*.

Each class was resolved into individual compounds by paper chromatography *(1, 6)*. For identification, spots were cut out from paper strips and extracted. The resulting fractions were identified by comparing R_F values run singly and mixed with authentic derivatives, and their absorption maxima in carbon tetrachloride and/or alcoholic alkali. Spectra in alcoholic alkali were useful in examining fractions separated on Vaseline impregnated paper. Although Vaseline can be removed from the fractions *(1)*, an impurity which absorbs at the lower wavelengths carries over. This interfered, particularly when the fractions were small. The fading rate in alcoholic alkali *(1, 8)* is also very helpful in clearing up doubtful cases. Spectrophotometric values obtained for authentic members of the four classes are shown in Table 2.

Identification is greatly simplified by the effectiveness of the separation into classes *(6)*. 2-Alkanone, n-alkanal, alk-2-enal, and alk-2,4-dienal are separated completely except for the acetone derivative which separates in the n-alkanal class, and methanal and ethanal in the alk-2-enal class. When the

TABLE 2

Spectrophotometric constants of carbonyl classes

Monocarbonyl classes	CCl_4	Alc. KOH		
	λ max.	Major λmax.		Minor λ max.
	mμ	mμ	Fading at 30 min.	mμ
2-Alkanones				
$C_3 - C_6$	349–52	430–34	1.2%	535
n-Alkanals				
C_1	330	420–6	69.0%	none
$C_2 - C_3$	343	430	42.0%	520
$C_4 - C_{11}$	343–6	430–4	27.0%	520
Alk-2-enals				
C_3	355	454	7.0%	none
C_4	361	454	2.8%	none
$C_5 - C_{12}$	361–5	456–60	1.7%	none
Alk-2,4-dienals				
C_5	370–5	470–4	1.9%	none
C_6	380	474–80	0.0%	none
$C_7 - C_{12}$	380	480–4	0.0%	none

classes are resolved into individual compounds, acetone forms mixtures with propanal and butanal, and ethanal with acrolein.

The identification of the monocarbonyls of the unheated and the heated fat is shown in Tables 3 and 4. In Table 3, the n-alkanal class contained propanal, hexanal, and nonanal; and the mixed class is shown to be composed of methanal and ethanal, three unknown compounds and $C_7 - C_{11}$ alk-2-enals. The constants of ethanal indicated that acrolein was not present. The first unknown compound with R_F 0.55 had spectra similar to n-alkanals but no secondary maximum in alcoholic alkali. The fading rate was also less than it should be for such a type of compound. Moreover, no n-alkanal would be expected in this position. The other two unknowns had properties intermediate between n-alkanals and alk-2-enals *(5, 7)*. No 2-alkanone would be present in these positions. These are apparently compounds of a different class, possibly unconjugated unsaturated compounds; although mixtures with C_5 and C_6 alk-2-enals might be involved. A similar group of three unknown compounds has been found in more oxidized fat *(7)*. Constants of propanal indicated the presence of little, if any, acetone. Traces of acetone, methyl ethyl ketone, methanal, and ethanal have been previously found *(5, 7)* as the result of benzene extraction following the carbon tetrachloride extraction. In this instance, however, no carbonyls were detected in the benzene extracts. The 2,4-dienal class contained the C_7, C_9, C_{10}, and C_{11} compounds. Identification of the carbonyls in the heated fat, shown in Table 4, presented no problems. The three unknown compounds, methanal, ethanal, propanal, and nona-2,4-dienal, present in unheated fat, were not detected in the heated sample. However, octanal was present in the heated fat, and was not observed in the unheated fat.

Table 5 shows the amounts of the classes and individual compounds found in 10.0 g. of fat in terms of absorbance, micro-moles, and micro-mole percent. The absorbancies of the classes are corrected values for monocarbonyls in

TABLE 3

Carbonyls in unheated fat

Unknowns R_F	CCl_4 λ max. mμ	Alcoholic λ max. mμ	KOH λ sec. max. mμ	Fading at 30 min.	R_F authentic compounds	Kind of carbonyl/ DNPH	Agreement w/constants of authentic
Saturated class							
Propylene glycol							
0.63	343–6	430–4	500–10 [1]	24.5%	C_8–0.64	n-alkanal	yes
0.92	346	430–4	500–10 [1]	25.0%	C_6–0.92	n-alkanal	yes
Vaseline system							
0.55	346	430–4	500–10 [1]	24.0%	C_9–0.55	n-alkanal	yes
Mixed class							
Propylene glycol							
0.21	330				C_1–0.21	2-enal	yes
0.38	343				C_2–0.38	2-enal	yes
0.55	343–6	428–36	none	16.4%	C_4–0.54	2-enal	no
0.71	352–5	440–48	none	7.0%	C_8–0.73	2-enal	no
0.80	352–5	444–50	none	6.1%	C_9–0.80	2-enal	no
Vaseline system							
0.68		450–58	none		C_7–0.68	2-enal	yes
0.62		450–58	none		C_8–0.62	2-enal	yes
0.57		450–58	none		C_9–0.57	2-enal	yes
0.52		450–58	none		C_{10}–0.52	2-enal	yes
0.42		450–58	none		C_{11}–0.42	2-enal	yes
2,4-Dienal class							
Propylene glycol							
0.70	380	465–80	none		C_7–0.70	2,4-dienal	yes
Vaseline system							
0.58		465–80	none		C_9–0.58	2,4-dienal	yes
0.52	375–80	465–80	none		C_{10}–0.52	2,4-dienal	yes
0.47		465–80	none		C_{11}–0.47	2,4-dienal	yes

[1] Disappeared after 30 min.

TABLE 4

Carbonyls in heated fat

Unknowns R_F	CCl_4 λ max. mμ	Alcoholic λ max. mμ	KOH λ sec. max. mμ	Fading at 30 min.	R_F authentic compounds	Kind of carbonyl/ DNPH	Agreement w/constants of authentic
Saturated class							
Propylene glycol							
0.91	346–9	430–9	500–10 [1]	25%	C_8–0.91	n-alkanal	yes
Vaseline system							
0.64		432	500–10 [1]	25%	C_8–0.64	n-alkanal	yes
0.58		432	500–10 [1]	25%	C_9–0.58	n-alkanal	yes
2-enal class							
Vaseline system							
0.73		452–6	none		C_7–0.73	2-enal	yes
0.67		452–6	none		C_8–0.67	2-enal	yes
0.60		452–6	none		C_9–0.61	2-enal	yes
0.52		452–6	none		C_{10}–0.52	2-enal	yes
0.45		452–6	none		C_{11}–0.46	2-enal	yes
2,4-dienal class							
Propylene glycol							
0.68	380	464–75	none		C_7–0.68	2,4-dienal	yes
Vaseline system							
0.59	380	464–80	none		C_{10}–0.59	2,4-dienal	yes
0.52		464–75	none		C_{11}–0.52	2,4-dienal	yes

[1] Disappeared after 30 min.

TABLE 5

Quantitative relationships of monocarbonyls

	Unheated fat						Heated fat					
	Corr. absorbance 10 g.	Percent based on absorbance	Micromoles per 10 g.	Percent micromoles (total)	Rel. parts absorbance	Micromoles per 10 g.	Corr. absorbance 10 g.	Percent based on absorbance	Micromoles per 10 g.	Percent micromoles (total)	Rel. parts absorbance	Micromoles per 10 g.
n-alkanals												
Methanal					0.100	0.022						
Ethanal					0.234	0.052					——	
Propanal					0.400	0.089					——	
Hexanal	0.642	72.3	3.01	82.2	12.86	2.808					12.97	2.133
Octanal					——	——	0.487	29.6	2.28	39.8	0.347	0.058
Nonanal					0.178	0.039					0.542	0.089
Unknowns												
1					0.063							
2	0.029	3.2	X	X	0.085							
3					0.072							
Alk-2-enals												
Hepta-2-enal					0.162	0.087					0.155	0.374
Octa-2-enal					0.140	0.076					0.135	0.325
Nona-2-enal	0.107	12.1	0.37	10.1	0.125	0.067	0.498	30.3	1.74	30.4	0.157	0.376
Deca-2-enal					0.162	0.087					0.141	0.340
Undeca-2-enal					0.097	0.053					0.133	0.325
Alk-2,4-dienals												
Hepta-2,4-dienal					0.088	0.010					0.353	0.032
Nona-2,4-dienal	0.110	12.4	0.38	7.7	0.110	0.012	0.660	40.1	1.71	29.8		
Deca-2,4-dienal					2.362	0.251					18.35	1.659
Undeca-2,4-dienal					0.070	0.007					0.211	0.019
Total	0.888		3.66				1.645		5.73			

100 ml. based on the recoveries (discussed in a preceding paragraph) in the class separations, and the fact that the mixed class from the heated monocarbonyls was found to be composed of 27.0% n-alkanal and 17.7% unknown class. Micro-moles were calculated on the basis that three micro-moles in 100 ml. of carbon tetrachloride were found to have average absorbancies of 0.640 for n-alkanals, 0.861 for alk-2-enals, and 1.160 for alk-2,4-dienals.

The qualitative and quantitative changes in the carbonyls on heating are of considerable interest. The fate of methanal, ethanal, propanal, the three unknown compounds, and 2,4-nonadienal on heating is not clear. Hexanal, propanal, and ethanal *(5)* have been detected in volatiles trapped during heating. The indication has been *(7)* that the amounts volatilized during heating are relatively very small. However, most of the compounds that disappear in the heated monocarbonyls were originally present only in trace amounts. In earlier experiments with more oxidized fat, propanal has carried over into the heated monocarbonyls *(7)*, but methanal has never been detected under these conditions. It is probable that such disappearances are due to both destruction during heating and volatilization. Hexanal and deca-2,4-dienal *(14)* were predominantly the major compounds in their respective classes in both unheated and heated fat. For instance, on a micro-mole basis, hexanal was 90.3% and 96.9% of the n-alkanal class, and deca-2,4-dienal 89.6% and 97.0% of the alk-2,4-dienal class respectively. This might cause failure to detect some trace amounts of monocarbonyls since the capacity of the filter paper is small. Hexanal was the major monocarbonyl in both the unheated and the heated fat. A similar observation was made by Klose *(9)* for rancid turkey fat. However, hexanal showed a decrease in the heated fat, while deca-2,4-dienal and the other unsaturated monocarbonyls increased considerably. The five alk-2-enals were similar in proportions.

Quantitative data given may be considered tentative in nature. The method for separation into classes is reproducible *(7)*. A mixture containing known quantities of the four classes, methyl ketones 24.6%, n-saturated aldehydes 20.8%, 2-enals 24.1%, and 2,4-dienals 30.5%, was found to contain 25.3%, 24.6%, 24.0%, and 26.1%, respectively *(6)*. Thus, values for 2,4-dienals may be somewhat low and the other classes high. The difficulty arises from the fact that the classes of compounds handled vary in stability. The alk-2,4-dienal hydrazones are particularly sensitive to light and air *(6)*. Further study is required on the quantitative limitations of the procedure used.

SUMMARY

Micro-methods of separating, identifying, and estimating monocarbonyls have been applied to a freezer-stored pork fat in the early stages of rancidity. Trace amounts of acetone and methyl ethyl ketone found in earlier studies with more oxidized pork fat were not observed. Heating at 165° C. influenced not only the amount, but the kind of monocarbonyls detectable. n-Alkanals C_1, C_2, C_3, C_6, C_9, three unknown carbonyls, alk-2-enals C_7, C_8, C_9, C_{10}, C_{11}, and alk-2,4-dienals C_7, C_9, C_{10}, C_{11} were found in the unheated fat. In the heated fat, n-alkanals C_6, C_8, C_9, alk-2-enals C_7, C_8, C_9, C_{10}, C_{11}, and alk-2,4-dienals C_7, C_{10}, C_{11} were observed.

Tentative quantitative determinations indicated the micro-mole content of the identified monocarbonyls. Hexanal and deca-2,4-dienal were the dominant compounds present.

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LITERATURE CITED

1. Ellis, Rex, Gaddis, A. M., and Currie, G. T. Paper chromatography of 2,4-dinitrophenylhydrazones of saturated aliphatic aldehydes. *Anal. Chem.*, **30**, 475 (1958).
2. Forss, D. A., Dunstone, E. A., and Stark, W. The volatile compounds associated with oxidized flavour in skim milk. *J. Dairy Research*, **22**, 91 (1955).
3. Forss, D. A., Dunstone, E. A., and Stark, W. Further observations on the volatile compounds associated with oxidized flavour in skim milk. *J. Dairy Research*, **22**, 345 (1955).
4. Forss, D. A. and Dunstone, E. A. Change of 2,4-dinitrophenylhydrazones on alumina and silica gel columns. *Australian J. Chem.*, **10**, (4) 506 (1957).
5. Gaddis, A. M. and Ellis, Rex. Volatile saturated aliphatic aldehydes in rancid fat. *Science*, **126**, 745 (1957).
6. Gaddis, A. M. and Ellis, Rex. Paper chromatography of 2,4-dinitrophenylhydrazones. The resolution of 2-alkanones, alkanal, alk-2-enal, and alk-2,4-dienal derivatives. *Anal. Chem.* **31**, 870 (1959).
7. Gaddis, A. M., Ellis, Rex, and Currie, George T. Carbonyls in oxidizing fat. I. Separation of steam volatile monocarbonyls into classes. *Food Research*, **24**, 283 (1959).
8. Jones, L. A., Holmes, J. C., and Seligman, R. B. Spectrophotometric studies of 2,4-dinitrophenylhydrazones. *Anal. Chem.*, **28**, 191 (1956).
9. Klose, A. A. (Unpublished data, 1952).
10. Lea, C. H. Recent developments in the study of oxidative deterioration of lipids. *Chemistry and Industry* (London) **41**, 1303 (1953).
11. Morris, S. G. Fat rancidity. Recent studies on the mechanism of fat oxidation in relation to rancidity. *J. Agr. Food Chem.* **2**, 126 (1954).
12. Moser, H. A., Joeger, C. M., Cowan, J. C., and Dutton, H. J. The flavor problem of soybean oils. II. Organoleptic evaluation. *J. Am. Oil Chemists' Soc.*, **24**, 291 (1947).
13. Pippen, E. L., Nonaka, M., Jones, F. F., and Stitt, F. Volatile compounds of cooked chicken. I. Compounds obtained by air entrainment. *Food Research*, **23**, 103 (1958).
14. Swift, C. E., O'Connor, R. T., Brown, L. E., and Dollear, F. G. The aldehydes produced during the autoxidation of cottonseed oil. *J. Am. Oil Chemists' Soc.*, **26**, 297 (1949).

AMINO ACID DISTRIBUTION IN GLUTEN FRACTIONS[a,b]

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Cereal chemists have long been interested in the cause of large variations in baking quality among wheat varieties of approximately equal protein content and grown under comparable environments. A large number of investigations have been carried out with the purpose of finding the basic reason for these differences, with interest centering on the gluten proteins and their properties.

Lusena *(9)* described a procedure for preparing wheat gluten for baking purposes based upon the separation of gluten dispersed in acetic acid by adjusting the pH to the iso-electric point with calcium hydroxide. Harris and Frokjer *(5)* modified Lusena's method to separate three fractions from gluten dispersion in lactic acid at pH levels of 5.0, 5.0 to 5.5 and over 5.5 by suitable additions of calcium hydroxide. The fractions were dried and added to soft wheat flour of low baking quality to bring the protein level to 13.0% (13.5% moisture basis.) Fraction II, precipitated at pH 5.5, was markedly superior to the other two fractions, comparing favorably with the original unfractionated gluten, but fraction I was very inferior. Fraction III yielded results resembling the soft wheat flour, indicating that little improvement was effected by the fraction. In the same study, durum wheat fractions produced the same results except that fraction II was a little lower in baking quality.

Harris and Sullivan *(6)* subsequently used pH levels of 5.2, 5.8, and 6.8 to increase the yield of protein precipitated. The properties of the fractions obtained were essentially identical with those separated by Harris and Frokjer.

Since Harris and Frokjer failed to find an adequate explanation for the marked differences in baking properties of the three fractions, it seemed that their amino acid distribution might be of interest. Hepburn, Lewis and Elvehjem *(7)*, however, found a nearly constant amino acid composition in the proteins of two blends of hard red spring wheat and two of hard red winter wheat. Also the amino acid distribution within the protein of the flours milled from these wheats tended to be the same although the degree of refinement ranged from 80 to 98.5% patent.

EXPERIMENTAL

Gluten preparation. Three flours of different baking quality were used for preparing the gluten. Their protein content, loaf volume, and dough mixing properties are shown in Table 1.

Essentially, the procedures described were followed in preparing the gluten fractions

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[a] Published with the approval of the Director.

[b] Condensed from a thesis presented to the Graduate School in partial fulfillment of the degree of Master of Science in Chemical Technology.

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TABLE 1

Quality properties of the three flours

Flour Code No.	Protein content	Loaf volume	Mixogram characteristics
	%	cc.	
55-113	12.6	725	Strong
53-98	12.4	715	Medium
55-136	10.9	595	Weak

from the flours. All glutens were washed with 0.1% sodium phosphate solution of pH 6.8 as described by Dill and Alsberg *(3)*; then after standing dispersed in 0.013*N* lactic acid with a Warning blender. The dispersion was centrifuged, and any residue removed. Saturated calcium hydroxide was used to adjust the pH for gluten precipitation. "Purified" gluten was prepared by a second precipitation and dispersion *(9)*.

Gluten hydrolysis. Satisfactory hydrolysis is required before an amino acid assay can be made of a protein. A number of methods to accomplish this are described in the literature. Stockelbach and Bailey *(13)* used 20% H_2SO_4, but a large amount of $BaSO_4$ was needed to remove the acid. Levy and Chung *(8)* employed 20% HCl sealed in a glass tube with the protein, followed by heating at 150° C. for 6 hours. Wellington *(14)* sealed the protein in a bomb with 50 times its weight of constant boiling HCl. Although both H_2SO_4 and HCl are equally efficient hydrolyzing agents for proteins, the latter is volatile and may be conveniently removed by vacuum distillation and desiccation *(12)*. For these reasons HCl was used in the present investigation.

The gluten was hydrolyzed as follows; 0.5 g. of crude gluten was placed in a 60 ml. round bottomed flask, fitted with a reflux condenser. Then 40 ml. of 5.913*N* HCl was added, and the mixture gently refluxed. After completion of hydrolysis the hydrolyzate was evaporated to dryness in an oven at 80° C. The residue was taken up with a little water and again dried. The amino acid hydrochlorides were dissolved in water and the solution adjusted to pH 4.0 with dilute NH_4OH. The solution was then centrifuged and the supernatant removed from the precipitated humin. This was washed once. The two two clear liquid layers were combined, evaporated carefully to reduce the volume and the hydrolyzate solution made up to 50 ml. with an isopropanol content of 10%. This amino acid solution was utilized for subsequent chromatographic analyses.

The biuret reagent specified by Pinckney *(10)* was first used to follow the progress of hydrolysis. This procedure, unfortunately, did not definitely indicate the completion of hydrolysis and was abandoned. The suitability of the ninhydrin reaction for this purpose was then investigated.

For this method, a solution of ninhydrin, 1.35 g. in 50 ml. of 95% ethanol, was used. The hydrolysis was traced by adding 1 ml. aliquots of hydrolyzate to 1 ml. of 0.069*N* KOH adjusted to 7.0 ± 0.2 with 0.05*N* acetic acid. Then 1 ml. of ninhydrin reagent was added, the solution diluted to 25 ml. in a 25 ml. volumetric flask and placed in boiling water for 3 minutes. The solution was next diluted, after cooling, to 500 ml. and let stand 20 hours for the color to develop. The transmittance was determined with a Beckman Model "B" Spectrophotometer at 420 and 580 mμ. The percent transmittance was compared to that of water. Representative data from this procedure are shown in Figure 1.

Chromatographic methods. Three methods of amino acid separation by paper chromatography were investigated before a satisfactory procedure was selected. Sheets of Whatman No. 1 filter paper marked "for chromatography" were employed. Solvents used were those employed by Davies, Harris and Parsons *(2)*. In all methods the solvent for the first run was prepared by mixing n-butanol, glacial acetic acid and distilled water in proportions of 25:6:25. The clear, organic layer remaining after shaking was used as the solvent. For the second run 95% ethanol and 2 *N* NH_4OH were used in the proportion 9:1.

The required amount of solution or hydrolyzate was applied 3 inches from each side near one corner of the paper with a micro-pipette in 3 μl portions. The spot was dried under a heat lamp between each application to restrict the spot to less than 1 cm. diameter. Before application to the paper 30% H_2O_2 solution equal to 1/5 the volume of the standard solution or hydrolyzate was added to the sample to oxidize cysteine and cystine

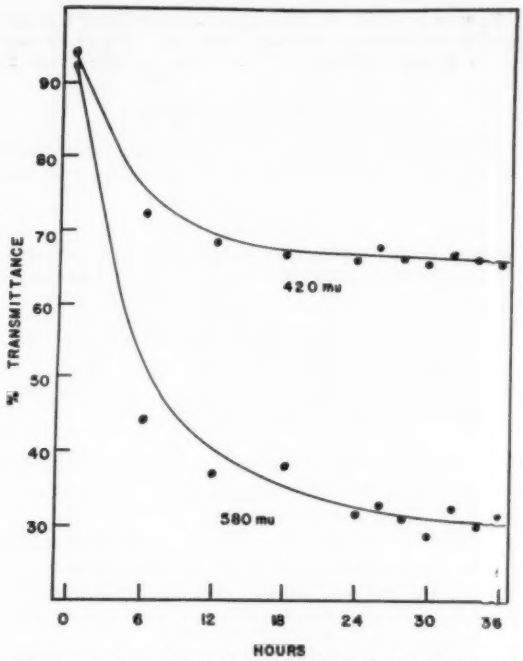

Figure 1. Representation of hydrolysis of wheat gluten by 5.913 N HCl. Degree of hydrolysis ascertained with the ninhydrin reagent.

to cystic acid and methionine to methionine sulfone. This required 4 to 5 hours for complete reaction *(14)*.

The spot was exposed to NH_4OH vapors for 5 minutes to neutralize any remaining HCl and free the amino acids. A 5-minute hydration over a steam bath completed the spot treatment. Glycine and serine as well as leucine and isoleucine were determined as one spot. Tryptophan was not determined because it was destroyed during acid hydrolysis. The eluate from the blank of the same size as the spot was used to obtain the zero setting of the slit opening.

Spot estimation followed the same procedure for the first 3 methods. The spots were eluted and the optical density of the eluate ascertained. Elution was accomplished by shaking in 50% aqueous, n-propanol, followed by removal of paper and lint. All optical densities were read at 570 mμ except for proline, which was read at 350 mμ.

In method No. 1, the dried chromatograms, after dipping in 0.5% ninhydrin-acetone solution, were dried in a cabinet for 30 hours at a temperature of 35° C. ± 1° and humidity 45–50%. For method No. 2, after dipping, the chromatograms were dried in a forced-draft oven at 70° C. for 10 minutes.

In method No. 3, the second irrigation differed from that used in methods No. 1 and No. 2. The solvent was allowed to flow only to the bottom of the paper; then the paper was removed from the tank and dried. Another run was made with the same solvent in the same direction as the last run for 12 hours. After air drying, the color was developed by dipping in ninhydrin-acetone solution and drying as in method No. 2.

A method for quantitatively estimating the acids by the area measurement recommended by Fischer, Parsons and Morrison *(4)* and Akerfeldt *(1)* was also used. Contoura prints from method No. 3 were used. Personal judgment was involved in deciding the exact boundaries, since spot edges were not sharply defined. Outlines of the spots were measured with a planimeter.

Solutions of 18 amino acids in the proportions that are known to occur in wheat gluten were prepared at six concentrations, and these were assayed by each of the four methods described, to obtain information on the most useful method for analyzing the glutens.

RESULTS AND DISCUSSION

Comparison of methods. Results of the analyses of the standard amino acid solutions by the four methods are shown in Table 2 as variance components for the 3 chief sources of variation. This analysis represents the proportion of the variability contributed by each of the 3 sources of variation after correction for residual variance or interaction and by the number of elements involved in each variance *(11)*. These variance components do not include the variance due to interactions. The components consisted of the replications, 4 in number, and the number and contentrations of amino acids, 15 and 6, respectively. Values represent the percentage of the total effect contributed by each component.

TABLE 2

Variance components for replications, amino acids and concentrations [1]

Component	Methods			
	1	2	3	4
Replications	0.0	0.3	0.2	0.5
Amino Acids	82.9	83.8	91.4	98.8
Concentration	17.1	15.9	8.4	0.7

[1] Expressed as % of variance contributed by main sources of variation.

The replication error was extremely low for all 4 methods, indicating very satisfactory reproducibility of the optial density reading. The largest part of the variability was contributed by the amino acids, and shows that all the methods differentiated satisfactorily between these. For detecting concentration differences methods No. 1 and No. 2 seemed the most sensitive, while method No. 4 was the least efficient although it seemed to be the most sensitive to amino acid identity. Method No. 3 was next sensitive. Methods No. 1 and No. 3 gave very high correlations between amino acid concentration and optical density values. Method No. 3 was finally selected for determining the amino acid content of the hydrolyzates because there was less variability in optical density readings for the main amino acids. This was particularly true with proline, which varied considerably in optical density in method No. 1. Figure 2 is a typical chromatogram prepared by method No. 3. Figure 3 represents the relation between amino acid concentrations and optical density for four amino acids and is typical of the results obtained with the other amino acids.

Amino acids in gluten fractions. Figure 4 represents the concentrations of glutamic acid and leucine + isoleucine between the glutens of the 3 flours. For the strong flour glutamic acid increased markedly from fraction I to III, while in the medium flour there was little difference between fractions II and III, although fraction III had the smallest amount. For the weak flour fractions I and III were approximately the same, while fraction II was markedly higher. The glutamic acid levels in fraction II of all three flours approximated that of the crude gluten of each. Also, there was a pro-

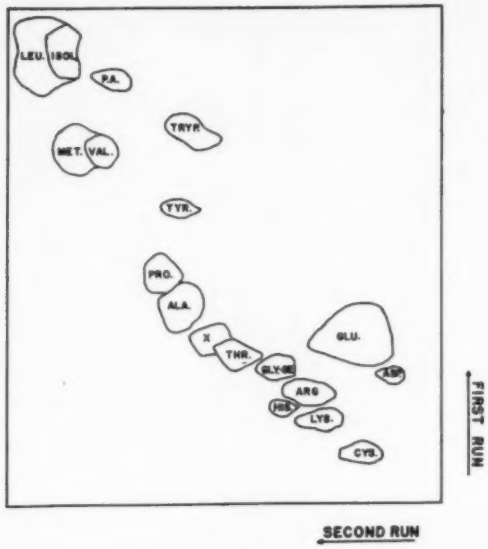

Figure 2. A typical chromatogram.

Key to abbreviations.

cysteic acid—cys	aspartic acid—asp	leucine—leu
arginine—arg	histidine—his	lysine—lys
glycine & serine—gly-se	threonine—thr	glutamic acid—glu
alanine—ala	proline—pro	"x"—unidentified
tryptophan—tryp	valine—val	tyrosine—tyr
methionine sulfone—met	isoleucine—isol	phenylalanine—p.a.

gressive decrease in the amino acid content of fraction III with the strong flour being highest followed in order by the medium and weak flours.

Differences in leucine + isoleucine were not as marked as for glutamic acid. Increasing amounts appeared in fraction I and fraction III in comparing the strong, medium and weak flours, respectively.

Glycine + serine and proline distributions are represented in Figure 5. For the strong and medium flours glycine + serine were much higher in fraction I, but for the weak flour they were highest in fraction III. They were lowest in fraction I for all the flours and showed a slight decrease from the strong to weak flour. It was highest in fraction II of the strong flour and in III of the medium flour. For these two flours, proline increased from fractions I to III. Proline content was lowest in the weak flour.

Figure 6 shows the results for lysine and arginine. For lysine the outstanding feature is the high proportion present in each flour in fraction I, with the highest in the weak flour. Differences between the remaining fractions were minor and without significance. There seemed to be no important differences between the original unfractionated glutens of the three flours. However, for arginine the medium strength flour appeared to be lowest, due possibly to the low amounts present in fractions II and III. Fraction I had the largest proportion in all the flours.

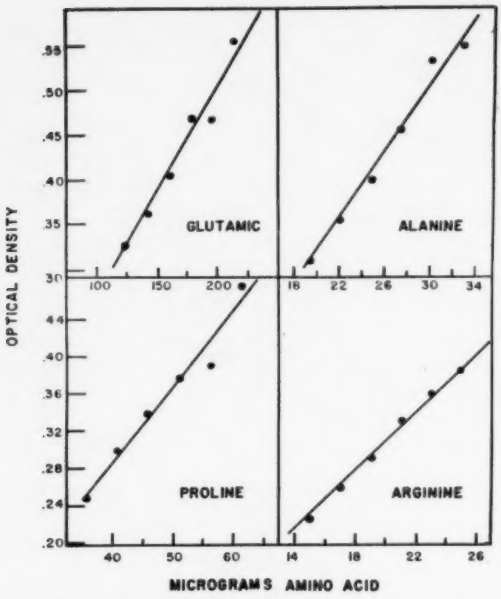

Figure 3. Standard curves for glutamic acid, alanine, proline, and arginine.

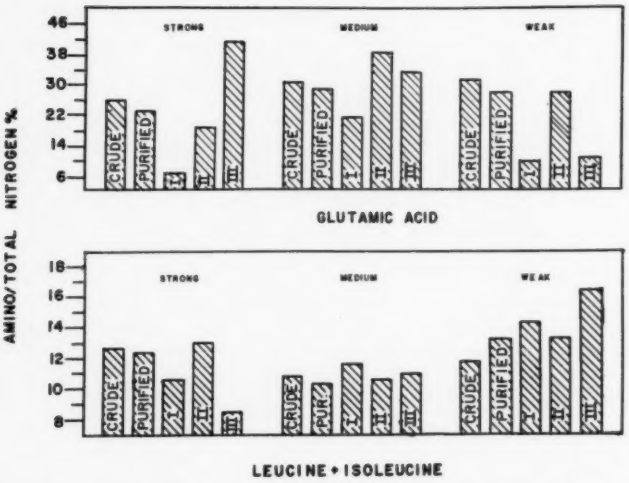

Figure 4. Bar graphs showing distribution of glutamic acid, and leucine + isoleucine.

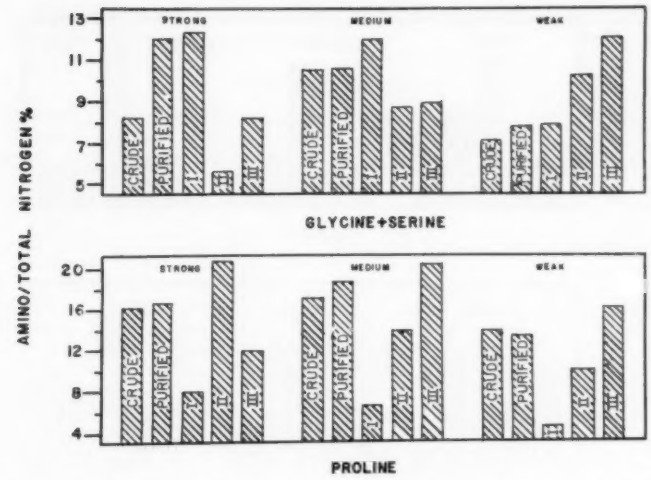

Figure 5. Bar graphs showing distribution of glycine + serine, and proline.

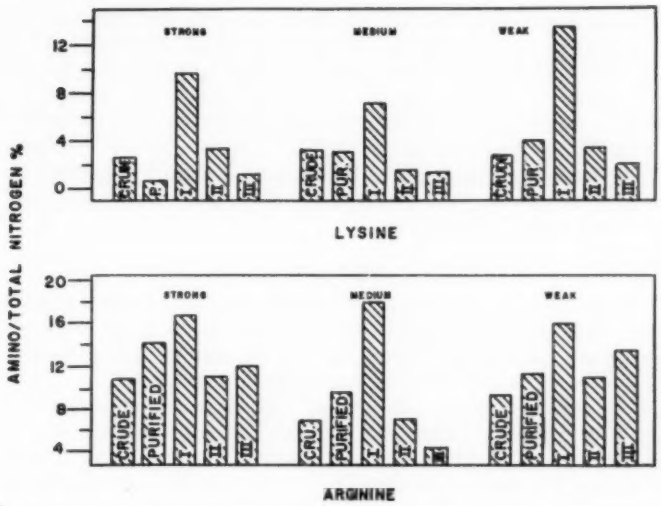

Figure 6. Bar graphs showing distribution of lysine and arginine.

Figure 7 provides information on the distribution of aspartic acid and alanine. There seemed to be no significant differences in the distribution of either acid among the flours, although possibly the medium sample contained slightly less alanine. Fraction I generally contained the highest amount of aspartic acid, and fraction III the lowest for the strong and medium flours.

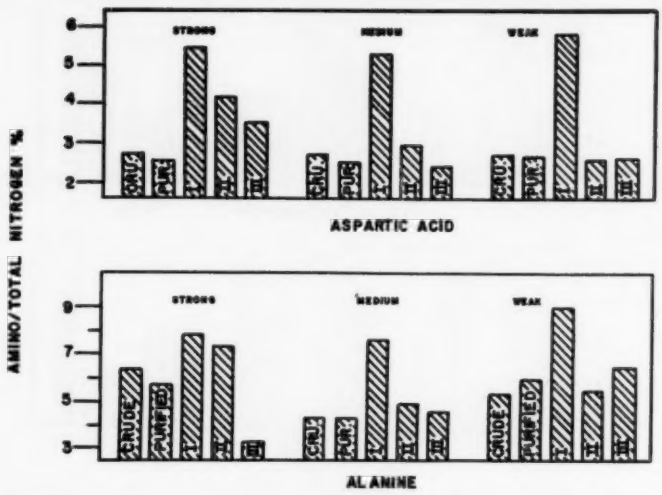

Figure 7. Bar graphs showing distribution of aspartic acid and alanine.

For the weak flour there was no difference between fractions II and III. Alanine also tended to be higher in fraction I, although there was little difference between I and II in the strong flour. However, it showed a progressive increase in fraction III from the strong to weak flour.

It is perhaps difficult to attempt to correlate the concentrations of these amino acids to baking behavior. However, there are trends as shown by fraction III indicating that high proportions of leucine + isoleucine, glycine + serine and alanine and low levels of glutamic acid and lysine might be related to poor baking properties.

SUMMARY

A two-dimensional paper chromatographic method suitable for the rapid separation and quantitative estimation of the amino acids of a protein hydrolyzate was developed. Three methods of separation and two of spot estimation were examined, and the most suitable selected.

Crude gluten, purified crude gluten, and three gluten fractions were prepared from each of the three flours which differed widely in their gluten strength characteristics. These protein preparations were then hydrolyzed, and the resulting hydrolyzates were analyzed for amino acid content by this chromatographic method. The results were calculated as percentages of the total nitrogen of the hydrolyzate contributed by each of the amino acids to obtain a common basis for comparison.

Significant variations existed between the amino acid levels of the different fractions and the flours. Since the main differences in glutamic acid, leucine + isoleucine, alanine and lysine levels among the three flours occurred in the third fraction, and since the differences in glycine + serine and proline distributions were in fractions II and III, it appeared that a more comprehen-

sive analysis of the third fraction of various flour glutens seems desirable to further define its relation to flour quality.

LITERATURE CITED

1. AKERFELDT, S. A spot area method for quantitative determination of amino acids on two-dimensional paper chromatograms. *Acta Chim. Scand.*, **8**, 521–522 (1954).
2. DAVIES, J. W., HARRIS, G., AND PARSONS, R. Nitrogenous constituents of brewing materials. *J. Inst. Brewing*, **62**, 38–51 (1956).
3. DILL, D. B. AND ALSBERG, C. L. Some critical considerations of the gluten washing problem. *Cereal Chem.*, **1**, 222–246 (1924).
4. FISCHER, R. B., PARSONS, D. S., AND MORRISON, G. A. Quantitative paper chromatography, *Nature*, **161**, 764–765 (1948).
5. HARRIS, R. H., AND FROKJER, DONALD. Gluten fractions of hard red spring and durum wheat and their baking quality. *Cereal Chem.*, **29**, 212–222 (1952).
6. HARRIS, R. H., AND SULLIVAN, J. W. Effects of gluten fractions on farinogram and and extensogram patterns. *Bakers Digest* **29**, 53–55 (1955).
7. HEPBURN, F. N., LEWIS, E. W., AND ELVEHJEM, C. A. The amino acid content of wheat, flour and bread. *Cereal Chem.* **34**, 312–322 (1957).
8. LEVY, A. L., AND CHUNG, DAVID. Two-dimensional chromatography of amino acids on buffered papers. *Anal. Chem.*, **25**, 396–399 (1953).
9. LUSENA, C. V. Preparation of dried native wheat gluten. *Cereal Chem.*, **27**, 167–178 (1950).
10. PINCKNEY, A. J. Wheat protein and the biuret reaction. *Cereal Chem.*, **26**, 423–439 (1949).
11. ROSANDER, A. C. *Elementary Principles of Statistics*. 1953. D. Van Nostrand Co., Inc. New York, N. Y.
12. SCHMIDT, CARL. *Chemistry of the Amino Acids and Proteins*. 1945. Charles C. Thomas, Springfield, Ill. pp. 123-133.
13. STOCKELBACH, L. S., AND BAILEY, C. H. The amino acid distribution in the proteins resulting from the thermal fractionation of wheat gluten. *Cereal Chem.*, **15**, 801–811 (1938).
14. WELLINGTON, E. F. The effect of relative humidity on the reaction of ninhydrin with amino acids on paper chromatograms. *Can. J. Chem.*, **31**, 484–487 (1953).

CHROMATOGRAPHIC IDENTIFICATION AND ESTIMATION OF THE FREE AMINO ACIDS PRESENT IN STRAWBERRY JUICE[a, b]

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As part of a study on the breakdown of strawberry anthocyanin in model systems, the effects of various normal constituents of strawberry juice on pigment loss were studied. Since Mackinney, Lukton and Chichester *(5)* have demonstrated that the rate of breakdown of the major anthocyanin of strawberries, pelargonidin-3-glucoside, in sugar or ascorbic acid systems was accelerated by the presence of glycine, it seemed reasonable to incorporate the effect of the amino acids of the strawberry into the experimental design. In order to determine the extent of this effect in strawberry products, it was necessary to know the identity and approximate concentration of the free amino acids present in the strawberry juice as it might be used under manufacturing conditions.

The only references to the amino acids of strawberry products are those of Casimir and Jakovliv *(3)* and Casimir *(2)*. These studies were made on acid hydrolysates and hence do not represent the free amino acids present in strawberry juice. This paper reports, therefore, the separation, identification and quantitative estimation of the free amino acids of the juice of the Marshall strawberry.

EXPERIMENTAL PROCEDURE

These experiments were carried out on a sample of juice obtained from Marshall strawberries which had been frozen and held for six months at 0° F. After thawing the juice was expressed through cheesecloth; a clear sample was obtained by further filtration through a sintered glass filter.

Qualitative studies. Hackman and Lazarus *(4)* have described a procedure for isolation of amino acids using one dimensional paper chromatography and a series of solvent systems. This procedure was followed using Whatman No. 1 filter paper. Using the single phase n-butanol/acetic acid/water system (77:6:17, v/v) and running the chromatogram twice in the same direction, the amino acids leucine and/or isoleucine, valine and alanine and sometimes glutamine were separated. Although not clearly separated in this solvent system the presence of aspartic acid and asparagine was also established by virtue of the characteristic colors produced with ninhydrin. Asparagine gave a brown color, while aspartic acid gave a blue-green color which turned purple on standing.

A single phase phenol/water system (74:26, phenol:buffer v/v) buffered with borate buffer at pH 10.0 gave a good separation of aspartic acid, glutamic acid, asparagine and threonine on paper buffered at pH 10.0. Alanine and glutamine were not separated in this system but alanine was separated in a preparation in which the glutamine and asparagine had been hydrolysed to glutamic and aspartic acids.

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[b] The data are from a thesis presented to the graduate school of Oregon State College by Ian J. Tinsley in partial fulfillment of the requirements for the degree Doctor of Philosophy.

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When a juice sample was developed in the phenol system, serine tended to run with the glutamic acid. To establish the presence of serine a sample of the juice was first chromatographed in the butanol system. After drying, the lower portions of the chromatogram were cut out and the amino acids eluted onto a buffered filter paper and then run in the phenol system. Serine was thus shown to be present in trace amounts.

Arginine was separated by first running the chromatogram in acetone buffered with phosphate buffer at pH 7.0 and then, after drying, running the chromatogram again in the same direction using 80% phenol as the solvent.

Because of the amount of dissolved material in strawberry juice and its effect on the Rf of the amino acids, direct comparisons with standard acids were not always possible. The presence of a particular acid was confirmed by the following procedure: Two aliquots of strawberry juice were applied to the same chromatogram and an additional aliquot of the amino acid under investigation was superimposed on one of the juice spots. After the chromatogram was developed an increase in spot size as well as color intensity was produced by the standard acid. Comparison with the untreated aliquot indicated whether the amino acid in question was present in the juice sample.

The presence of asparagine and glutamine was confirmed by the comparison of the aspartic acid and glutamic acid spots from hydrolyzed and non-hydrolyzed samples. To hydrolyze asparagine and glutamine, the juice was made 2 normal with respect to hydrochloric acid and then heated on a boiling water bath for 3 hours. The hydrolyzed sample was evaporated to dryness under vacuum and then placed in a desiccator over solid sodium hydroxide. For analysis the residue was taken up in the required amount of water and chromatographed.

Before chromatographic analysis, cysteine and cystine were converted to cysteic acid. The aliquot of juice was treated on the chromatographic paper with 30% hydrogen peroxide and ammonium molybdate, as described by Block, Durrum and Zweig (*1*, p. 94). Cysteic acid was identified in the buffered phenol system. No differentiation was made between cysteine and cystine.

Quantitative studies. When the amino acids had been identified and a satisfactory separation achieved, those present in larger amounts were estimated quantitatively. The method described by Moore and Stein *(6)* was adapted for this purpose. A standard curve was obtained by adding 0, 10, 30, and 40 μl aliquots of 2×10^{-3} M leucine (258 mg./liter) to pieces of Whatman No. 1 filter paper (3 x 2 cm.) which had been washed in distilled water. Each of these was placed in a test tube to which was added 0.5 ml. of water and 2 ml. of ninhydrin reagent. The ninhydrin reagent was prepared by adding 0.80 g. of stannous chloride dihydrate dissolved in 500 cc. of citrate buffer at pH 5 to 20 g. of ninhydrin in 500 cc. of methyl cellosolve. The tubes were covered with aluminum caps and the color developed by heating on a boiling water bath for 20 minutes. Five ml. of a mixture of n-propyl alcohol and water (1:1) were added and the absorbance measured at 570 mμ using a Beckman Model D.U. Spectrophotometer. A straight line relation between absorbance and concentration was obtained over this concentration range.

The amino acids were estimated by chromatographing a sample of the juice sufficient to give a concentration falling within the range of the standard curve. Three or 4 aliquots of equal size and an indicator aliquot were run 1 cm. apart on the same paper. After the chromatogram had been developed, the indicator strip was cut from the chromatogram, the color developed with ninhydrin and the spots on this strip used to position those to be cut out for analysis. These strips together with blanks of equal size were washed several times with ethyl ether to remove the solvent. The color was then developed in the same manner as that described for the standard curve. Moore and Stein *(6)* give factors which relate the color yield of different amino acids to that of leucine; hence, only one standard curve was required.

RESULTS AND DISCUSSION

The results obtained are summarized in Table 1. The major amino acids present in the free state are asparagine, glutamine, alanine, glutamic acid and aspartic acid. Those present in trace amounts were serine, threonine, arginine, valine, cystine and/or cysteine, and leucine and/or iso-leucine.

TABLE 1

Concentrations and Rf's of free amino acids in strawberry juice

Amino acid	Concentrations (mg./100 ml.)	Mean (mg./100 ml.)	Rf BuOH-HOAc-H_2O (77:16:17 v/v)	Rf Phenol—H_2O 74:26 borate buffer pH 10.0
Asparagine	64.7	59.4	.07	.39
	56.8			
	56.8			
Glutamine	17.1	14.5	.07	.55
	13.7			
	12.7			
Alanine	10.8	12.1	.22	.53
	13.3			
Glutamic acid	8.3	7.7	.15	.25
	9.7			
	5.2			
Aspartic acid	2.6	2.8	.09	.15
	3.0			
Serine	all < 2	< 2	.12	.31
Threonine	do	do	.16	.45
Valine	do	do	.46	....
Leucine/isoleucine	do	do	.60	....
Cysteic acid	do	do	....	.06

Since these experiments were carried out on juice from strawberries held frozen at 0° F. for six months, one cannot conclude that the free amino acid content of a fresh juice would coincide exactly with the results described in this paper. It is possible that some changes may have occurred in the free amino acid composition on storage even at these low temperatures.

SUMMARY

As part of a study on pigment loss in model systems, the free amino acids of the juice of Marshall strawberries, which had been held at 0° F. for six months, were determined. By co-chromatographing in one direction with knowns, using Whatman No. 1 filter paper and various solvents, the following were found: asparagine, glutamine, alanine, glutamic acid, aspartic acid, serine, threonine, arginine, valine, cystine and/or cysteine, and leucine and/or isoleucine. Concentrations ranged from 59 mg./100 ml. to less than 2 mg./100 ml.

LITERATURE CITED

1. Block, R. J., Durrum, E. L., and Zweig, G. *A Manual of Paper Chromatography and Paper Electrophoresis.* 1956. New York, Academic Press.
2. Casimir, J. Etude des acides aminés dans les fruits en conserve. *Bull. inst. agron. et sta. recherches Gembloux.*, **20**, 33. (1952).
3. Casimir, J. and Jakovliv, G. Etudes des acides aminés dans les fruits surgeles, les conserve de fruits et les confitures. *Inds. agr. et aliment.*, **69**, 787 (1952).
4. Hackman, R. H., and Lazarus, M. Quantitative analysis of amino acids using paper chromatography. *Australian J. Biol. Sci.*, **9**, 281 (1956).
5. Mackinney, G., Lukton, A., and Chichester, C. O. Strawberry preserves by a low temperature process. *Food Technol.*, **10**, 427 (1956).
6. Moore, S., and Stein, W. H. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.*, **176**, 367 (1948).

THE CAROTENOIDS OF CLING PEACHES

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Carotenoids of peaches were investigated chromatographically by Mackinney in 1937 *(15)*, using powdered vacuum-dried fruit of the Muir Variety. Eight bands were obtained, five of which were identified as zeaxanthin, lutein, cryptoxanthin, β-carotene, and α-carotene (a faint trace). Thaler and Schulte *(20)* in 1940 reported the carotenoid mixture of European yellow peaches to consist of eight parts lutein, two of carotene, and one of lycopene. The colorless polyenes, phytofluene, and phytoene, were found in peaches by Zechmeister and Sandoval *(25)*, and by Rabourn and Quackenbush *(18)*, respectively. Joyce *(8)* reported the presence in peaches of zeta-carotene. He also found that, unlike apricots, peaches contain lycopene and poly cis compounds in very small amounts, if present at all. McCarty and Lesley *(14)* reported the probable presence in peaches of α- and β-carotenes, cryptoxanthin, "isolutein," zeaxanthin, and phytofluene. The carotenoids of cling peaches have now been investigated, using countercurrent distribution in a glass Craig apparatus to separate the carotenoids into fractions, followed by chromatography to obtain the individual constituents. The pigment mixture was qualitatively similar to that obtained from Valencia orange juice *(2, 5)* but even more complicated. The major components were violaxanthin, cryptoxanthin, β-carotene, phytoene, and an apparently new xanthophyll for which the name "persicaxanthin" is proposed.

EXPERIMENTAL

Cling peaches of the Halford variety were obtained from a commercial cannery; the fruit had been halved, peeled, and pitted. Halves which were firm, bright orange yellow in color, and with no brown or green spots, were packed into cans which were then filled with a sirup containing 40% sucrose and 0.015% ascorbic acid, sealed, and transferred promptly to storage at −10° F. (−23° C.). When used, cans were allowed to thaw out overnight at 45° F. (7° C.), the sirup decanted off, and the halves rinsed with tap water. Only halves showing no browning were used.

In preliminary work the fruit was dehydrated with methanol, a procedure frequently employed in carotenoid extraction *(23, 24)*, and used by the present worker for citrus peels *(4, 6, 7)*. It was found that with this method the carotenoid 5,6-epoxides were completely isomerized to 5,8-epoxides (referred to in earlier publications by the present author as epoxides and furanoxides, respectively) by the acid in the peaches. Hence the following procedure, which was similar to that used for the edible portion of citrus fruits *(4, 7)*, was employed in further work.

Peaches in 250 g. portions were blended with 250 ml. of water plus 4 g. of calcium carbonate. The blendate was mixed with an equal volume of methanol, filter aid was added (20% by weight of the peaches used), and after about 30 minutes the mixture was filtered on a Büchner funnel precoated with filter aid. The filter cake was blended briefly with acetone, filtered on a sintered glass funnel, and the filter cake washed with acetone until the washings were apparently colorless. The acetone extract was worked up in the usual manner *(2)*; both unsaponified and saponified extracts were prepared.

Countercurrent distribution runs in an all-glass Craig apparatus were made with saponified (commonly referred to, in fat saponification, as the unsaponifiable fraction) and unsaponified extracts from 500–1500 g. of peaches. The countercurrent distribution

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runs were carried out in dim light as rapidly as possible (usually less than one minute per transfer is required). The solutions were removed as completely as possible from the tubes of the Craig apparatus by means of a glass syringe provided with a metal tip. It is not practicable to try to recover the residual material adhering to the tubes of the Craig apparatus.[b]

System I (petroleum ether and 99% methanol, 1.8 to 1 by volume), with 100 transfers, was used to separate the saponified pigments into: (1) hydrocarbons; (2) monols plus monol epoxides; and (3) diols plus polyols. With unsaponified carotenoids fraction (1) contained also xanthophyll esters *(3)* and fraction *(2)* probably also contained incompletely esterified xanthophylls. System II (benzene, petroleum ether, and 87% methanol, 1 to 1 to 1.15 by volume), with 199 transfers, was used to separate the saponified carotenoids into: (1) hydrocarbons plus monols; (2) diols; (3) monoepoxide diols; (4) diepoxide diols; and (5) polyols. The color in each (100-transfer runs) or every other (199-transfer runs) tube of the Craig apparatus was measured in an Evelyn photoelectric colorimeter, after addition of acetone to make the two phases homogeneous and to 25 ml. (System I) or 30 ml. (System II). Filter 440 was used and the results were calculated as β-carotene *(2)*. The contents of the tubes corresponding to the several fractions were then combined, evaporated in vacuo, and the residues dissolved in petroleum ether (hydrocarbons and monols) or benzene.

The 6 fractions obtained by countercurrent distribution (nos. 1 and 2 with System I, nos. 2-5 with System II) were chromatographed on columns of magnesia (Westvaco No. 2642) plus filter aid (1 to 1 by volume) *(2)*, 12 by *ca* 160 mm., and the constituents fractionated by a graded series of eluants, starting with, for hydrocarbons, 14% benzene in petroleum ether. For monols, diols, monoepoxide diols, diepoxide diols, and polyols, 0.3, 3.5, 3.5, 3.5, and 7% ethanol in petroleum ether, respectively, were employed.[c]

Subsequent eluants were *ca* 1.5 x as strong as the preceding one, resulting in a series such as 3.5, 5, 7, 10, 15, and 25% ethanol in petroleum ether. In the diepoxide diol frac-

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[b] Recoveries of material as measured at 440 mμ generally run about 97%, which includes mechanical losses due to incomplete removal of solutions from the tubes of the Craig apparatus and any losses due to atmospheric oxidation which may have occurred during the countercurrent distribution run, the sample solution removal, color measurement, and subsequent carotenoid recovery. Carotenoid losses due to atmospheric oxidation are therefore negligible during the countercurrent distribution procedure used in the fractionation of the carotenoids.

Many purified carotenoid constituents, obtained from leaves, oranges, and peaches by preliminary fractionation by countercurrent distribution, followed by chromatography, have been subjected to a second countercurrent distribution with system I or II (see below) in order to determine the N_{100} value *(2)*. In no case was there any significant amount of color elsewhere than in the main fraction. The polyol fraction therefore does not consist of artifacts formed from other carotenoids during the countercurrent distribution procedure. In a number of cases (including violaxanthin which is one of the more labile carotenoids) the recovered product was rechromatographed, and in no case was there any significant amount of substance recovered other than the starting material except for cis or trans isomers thereof. Cis-isomers (usually monocis, occasionally dicis) were sometimes formed in appreciable amount from trans isomers and vice versa, so that no significance is to be attached to the isolation of cis-isomers as they may be entirely artifacts. Cis-isomers are known to occur in plants as such, however; polycis compounds such as prolycopene or pro-γ-carotene *(22)* are not known to be artifacts. It was found that the acid-catalyzed 5,6-epoxide–5,8-epoxide isomerization (as of violaxanthin or antheraxanthin) did not occur to an appreciable extent during countercurrent distribution.

[c] In some cases bands obtained by chromatography on magnesia were rechromatographed on lime *(16)*. By this means a better separation of the all-trans and cis constituents can usually be obtained. Because the countercurrent distribution procedure may result in the formation of appreciable amounts of cis-isomers, separation of the latter is of no particular significance in the present investigation where the primary purpose was to identify the carotenoids present in peaches. Rechromatography on lime was not generally carried out due to the large number of bands (ca 45) obtained by the chroma-

tion 20% ethanol in benzene was used as the final eluant. Spectral absorption curves were run in a Cary recording spectrophotometer.

RESULTS AND DISCUSSION

The total carotenoid content of the saponified extract was equivalent to 27 mg. per kg. of fruit (as β-carotene, measured at 440 mμ in the Evelyn colorimeter). The countercurrent distribution of the saponified carotenoids with System II is shown in Figure 1. Based upon the countercurrent distribution in the two solvent systems, the composition of the carotenoid mixture (measured at 440 mμ and calculated as β-carotene) was: hydrocarbons 17%; monols 16%; monoepoxide monols 1%; diols 8%; monoepoxide diols 8%; diepoxide diols 42%; and polyols 7%. This method of measuring the carotenoid content is approximate only; colorless substances such as phytoene, phytofluene and the persicachromes are not measured at all, while other substances which absorb light at low wave lengths, such as persicaxanthin, zeta-carotene and auroxanthin, give misleadingly low values.

On chromatography, a total of 45 bands was obtained (Table I), which had spectral absorption curves indicating pure or rather pure substances. Eleven of these were apparently cis-isomers of other bands, and may have been entirely artifacts.

Hydrocarbon fraction (I). Eleven bands were obtained from this fraction (Table I), including 3 which were apparently monocis isomers. The phytofluene band on chromatography separated, though not widely, into 2 roughly equal bands, apparently monocis and all-trans isomers *(12)*. The native form in tomatoes is the cis-isomer *(17)*. Alpha-carotene was found as a very minor fraction, in agreement with earlier work *(15)*. Above the zeta-carotene band on the column was a yellow band (I-8, Table I) with absorption maxima corresponding to those of mutatochrome (β-carotene 5,8-epoxide), which was reported by Karrer and Jucker *(9, 10)* as a constituent of orange peel.

The next two bands, I-9 and I-10, had absorption maxima at wave lengths somewhat higher than β-carotene; both had cis-peaks at 349 mμ. On iodine isomerization *(1)*, the maxima of the principal peaks of both fractions shifted to 488 and 458 mμ, indicating this pair of bands to be mono-cis (I-9) and all-trans (I-10) isomers. These substances may be the same as Trombly and Porter's pigments B and C *(21)*, which were obtained from tomatoes, and which were shown to be distinct from γ- and δ-carotenes. The spectral absorption curve of fraction I-11 resembled somewhat that of prolycopene *(22)*, having humps at 497 and 463 mμ, with the principal maximum at 443 mμ, in petroleum ether; after iodine isomerization peaks were present at 499, 469, 444, and 361 mμ, indicating fraction I-11 to be a polycis lycopene.

Monol plus monoepoxide monol fraction (II). The monol fraction had

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tography on magnesia of the 6 fractions obtained by countercurrent distribution. Carotenoid pairs such as lutein and zeaxanthin, cryptoxanthin and "hydroxy-α-carotene," α- and β-carotenes, and phytofluene and α-carotene, are readily separable on magnesia columns. Cis-isomers of carotenoid epoxides are sometimes readily separable from the corresponding all-trans isomers, but other cis-isomers, especially of non-epoxide carotenoids such as β-carotene, cryptoxanthin, and lutein, are often not readily separable on magnesia. Due to its much greater capacity, magnesia was preferred for the chromatography of the 6 main fractions.

TABLE 1

Carotenoids obtained from cling peaches

Fraction	Constituent	Solvent	Spectral absorption maxima, mμ	Approximate percentage[2]	
I-1	Phytoene	P	(300), 289, 280	10	
I-2	cis-Phytofluene	P	367, 348, 332	1.2	2.3
I-3	Phytofluene[1]	P	368, 349, 333	1.1	
I-4	α-Carotene	P	472, 440, 413	0.1	
I-5	β-Carotene	P	478, 451, (426), 338	8.5	10
I-6	Neo-β-Carotene U[1]	P	474, 446, (422), 337	1.5	
I-7	Zeta-Carotene	P	425, 401, 379, 300	1.2	
I-8	Mutatochrome-like	P	453, 426, (402)	0.3	
I-9	Substance 485[1]	P	485, 455, 431, 350	0.3	
I-10	Substance 490	P	490, 460, 436, 350	0.5	
I-11	Polycis-lycopene	P	(497), (463), 443, 360	0.3	
II-1	"Hydroxy-α-Carotene"	P	474, 445, 420	0.2	
II-2	cis-Cryptoxanthin[1]	P	470, 444, (422), 335	0.9	12
II-3	Cryptoxanthin	P	478, 451, (427), 339	10.6	
II-4	cis-Cryptoxanthin[1]	P	473, 446, (425), 340	0.6	
II-5	cis-Cryptoflavin-like[1]	P	450, 427, (403)	0.3	
II-6	Crytoflavin-like	P	454, 428, (405)	0.5	
II-7	Rubixanthin-like	P	(490), 459, (428)	0.3	
IIIA-1	Lutein	B	486, 457, 433, 339	1.3	
IIIA-2	Zeaxanthin	B	492, 464, (438), 346	3.8	
IIIB-1	Ketonic (?) Carotenoid	B	423	0.2	
IIIB-2	Lutein 5,6-epoxide	B	483, 452, 426, 337, 322	0.2	
IIIB-3	Antheraxanthin	B	488, 458, (433), 339	3.5	
IIIB-4	Flavoxanthin-like	B	461, 433, 410	0.1	
IIIB-5	Mutatoxanthin a	B	465, 438, (415), 320	0.5	1.1
IIIB-6	cis-Mutatoxanthins[1]	B	463, 436, (412), 320	0.2	
IIIB-7	Mutatoxanthin b	B	465, 438, 415	0.4	
IIIC-1	Violaxanthin	B	483, 453, 427, 336, 321	15	26
IIIC-2	cis-Violaxanthin[1]	B	479, 449, 423, 336, 322	11	
IIIC-3	Luteoxanthin a	B	461, 433, 408, 319, 306	1.9	5.3
IIIC-4	Luteoxanthin b	B	461, 432, 408, 320, 307	1.5	
IIIC-5	cis-Luteoxanthin a[1]	B	458, 430, 406, 318, 305	1.1	
IIIC-6	cis-Luteoxanthin b[1]	B	457, 430, 406	0.8	
IIIC-7	Auroxanthins	B	436, 410, 377	0.7	
IV-1	Persicaxanthin	B	402, 380, 361	12	
IV-2	Trollixanthin-like a	B	480, 450, 426, 336, (320)	1.2	
IV-3	Trollixanthin-like b	B	482, 452, 427, 336, (320)	2.5	3.1
IV-4	Trollein-like	B	486, 456, 430	0.3	
IV-5	cis-Trollixanthin-like[1]	B	477, 447, 423, 335, 320	0.6	
IV-6	Trollichrome-like a	B	457, 431	0.2	
IV-7	Substance 402	B	402, 379, 360	0.3	
IV-8	Trollichrome-like b	B	458, 431, 406	0.6	
IV-9	Persicachrome a	B	373, 353, 336	0.5	
IV-10	Persicachrome b	B	373, 354, 337	0.7	
IV-11	Substance 335	B	335, 319, 306	0.5	

[1] Probably artifacts.
[2] Based upon total absorbance of each constituent at the wave length of the principal maximum. Values in parentheses represent humps or shelves on spectral absorption curves.
P = Petroleum ether: B = Benzene.

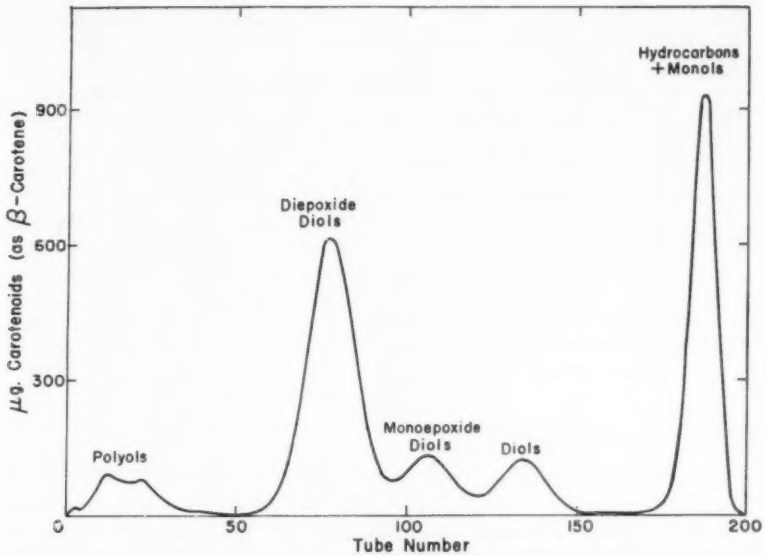

Figure 1. Countercurrent distribution of saponified carotenoids from cling peaches with the system benzene, petroleum ether, and 87% methanol.

an N_{100} value in system I of 57; a much smaller fraction corresponding to monoepoxide monols had an N_{100} of 38. The latter fraction was so small in amount (ca 1% of the total carotenoids) that it was collected and chromatographed together with the monol fraction. The constituents of this fraction resembled those obtained from oranges *(2, 6)*, except for the presence of a minor fraction highest on the column which resembled rubixanthin (hydroxy-γ-carotene). Cryptoxanthin was the principal monol while "hydroxy-α-carotene" was quite minor.

Diol fraction (IIIA). Lutein and zeaxanthin only were found, the latter in about 3 times the quantity of the former.

Monoepoxide monol fraction (IIIB). The principal component (IIIB-3, Table 1) of this fraction was apparently antheraxanthin (zeaxanthin 5,6-epoxide). The spectral absorption curve (Figure 2) resembled that of the antheraxanthin fraction from orange juice, also lutein which has the same conjugated double bond system as antheraxanthin. Antheraxanthin is readily distinguished from lutein by its different N_{100} value in System II (55 and *69*, respectively); it gives a light blue color in the hydrochloric acid-ether test and on treatment with acid is converted to mutatoxanthins. Three bands closely resembling the mutatoxanthin fractions of orange juice *(5)* were also obtained. These are isomeric zeaxanthin 5, 8-epoxides.

A minor band (III-2) was found on the column considerably below antheraxanthin, which was eluted more rapidly than lutein under the same conditions. The spectral absorption curve (Figure 3) resembled that of violaxanthin closely, even in the position of the two cis-peaks, so that the

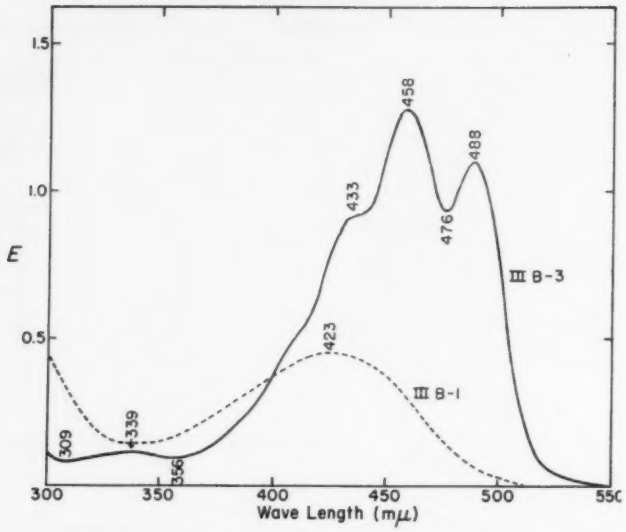

Figure 2. Spectral absorption curves in benzene of fractions III B-1 and III B-3 (antheraxanthin).

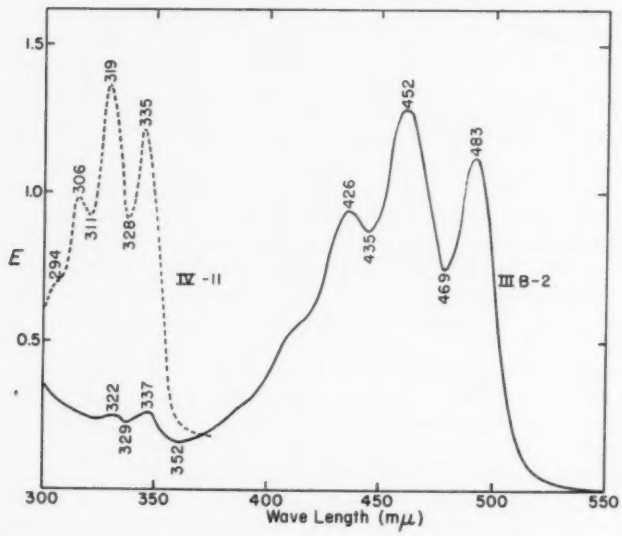

Figure 3. Spectral absorption curves in benzene of fractions III B-2 (lutein 5,6-epoxide) and IV-II.

two must contain the same conjugated double-bond system. In a mixed chromatogram with violaxanthin, fraction IIIB-2 was eluted about twice as rapidly. On comparison with violaxanthin in the hydrochloric acid-ether test *(5)*, the latter gave a clear deep blue, while IIIB-2 gave a much paler greenish-blue, indicating the presence in the latter of only one epoxide group. From this and from the spectral absorption curve and the behavior on countercurrent distribution and chromatography, it was concluded that this fraction was probably lutein 5,6-epoxide *(11)*. The occurrence on the column above antheraxanthin of a minor band resembling flavoxanthin (the isomeric lutein 5,8-epoxide) is supporting evidence for this identification, because in carotenoids obtained from acid fruits, 5,6-epoxides are usually accompanied by smaller amounts of the isomeric 5,8-epoxides.

Diepoxide diol fraction (IIIC). The main constituent of this fraction was identified as violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide), and was accompanied by a cis-isomer and by much smaller amounts of the isomeric 5,6,5′,8′-diepoxides (luteoxanthins, *5*), and still smaller amounts of the 5,8,5′,8′-diepoxides (auroxanthins).

A minor component (IIIB-1) was the first band eluted from both the monoepoxide diol and diepoxide diol fractions; the maximum for this substance on countercurrent distribution fell about halfway between the maxima for these *2* fractions. It gave no blue color in the hydrochloric acid-ether test *(5)*. The spectrophotometer curve (Figure *2*) showed the presence of one broad peak only; this was practically unchanged on iodine isomerization *(1)*, indicating that it was not a polycis compound such as pro-γ-carotene or prolycopene *(22)*. It may be a ketonic carotenoid such as canthaxanthin *(19)* or astacene *(13)*, but containing only about 8 conjugated double bonds.

Polyol fraction (IV). The chromatography of this fraction was not as clean-cut as for the others because of its greater complexity, as was the case also for orange juice carotenoids. In Figure 1 the presence on countercurrent distribution of two peaks in the polyol fraction (IV) indicates the occurrence of two substances or groups of substances with appreciably different partition coefficients, such as triols and tetraols. As in the monoepoxide diol and diepoxide diol fractions, the 5,6-epoxides were found in considerably greater amounts than the corresponding 5,8-epoxides.

The first band eluted (IV-1) resembled the first band obtained from the corresponding fraction from oranges, for which the name "valenciaxanthin" was proposed *(5)*. This band occurred in much greater amounts in peaches than did valenciaxanthin in oranges. It is not readily observed on the column because of the low wave length of the spectral absorption maxima (402, 380, 361 mμ, in benzene). Like valenciaxanthin it can be detected on the column more readily by its rather weak orange fluorescence in ultraviolet light. Two bands (IV-9 and IV-10) having a brilliant green fluorescence in ultraviolet light, like that of phytofluene and valenciachromes a and b, were found. Valenciachromes a and b are 5,8-epoxide isomers of valenciaxanthin, which is a 5,6-epoxide. Fractions IV-9 and IV-10 were eluted from the column more slowly than valenciachromes a and b.

On countercurrent distribution with the system petroleum ether, acetone, methanol, and water (1.25 to 1 to 0.1 to 0.65 by volume, a good system

for the fractionation of carotenoid polyols) valenciaxanthin had an N_{100} value *(5)* of 50, while that for peach fraction IV-1 was 39–40. The value for valenciachromes a and b (combined) was *ca* 58, while that of combined IV-9 and IV-10 was 48. This shows that the substances from peaches and oranges were different. Fraction IV-1 and valenciaxanthin are similar in that both are 5,6-epoxides and have very similar spectral absorption maxima, indicating the same conjugated double-bond system. Fraction IV-1 is more hydrophilic than valenciaxanthin. Because fraction IV-1 is one of the major carotenoids of cling peaches, the name "persicaxanthin" is proposed; the corresponding 5,8-epoxide isomers are named "persicachrome a" and "persicachrome b". The spectral absorption curves of persicaxanthin and persicachrome (combined a and b) are given in Figure 4.

The second band (IV-2) was well defined and well separated. and resembled the trollixanthin-like fraction from oranges *(5)* but was somewhat more readily eluted. Fraction IV-3 was apparently the same as the trollixanthin-like fraction from oranges. Fraction IV-7 had spectral absorption maxima very close to those of persicaxanthin and valenciaxanthin; a much stronger eluant was required hence it probably contains more hydrophilic groups, perhaps one more hydroxyl. The last band (IV-11) had spectral absorption maxima corresponding to a system of 4 conjugated double bonds. The spectral absorption curve (Figure 3) of this substance is carotenoid-like, of the type where none of the conjugated double bonds are in an ionone ring.

Further studies on the polyol constituents of peaches, oranges, and leaves are in progress.

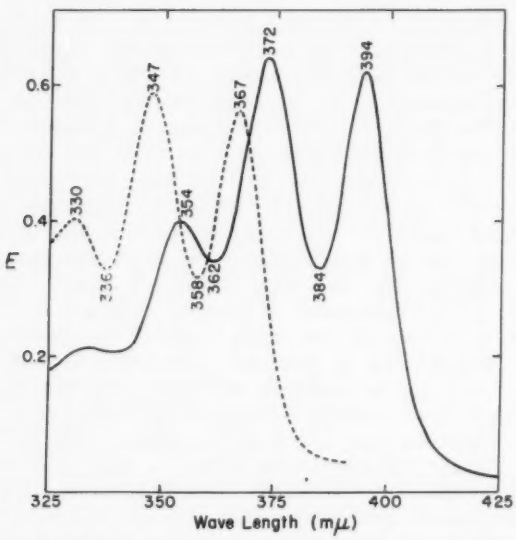

Figure 4. Spectral absorption curves in benzene of persicaxanthin (solid curve) and persicachromes (dashed curve).

Composition of unsaponified carotenoids. Countercurrent distribution of the unsaponified carotenoid extract with system I showed that, as in oranges *(3)*, most of the xanthophylls occurred as esters; 77% of the pigment occurred in fraction I as compared with 17% (the hydrocarbons) after saponification. On saponification of the unsaponified carotenoid extract, there was a loss of about 3% in total color (measured at 440 mμ), due at least partially to the formation of alkali-soluble pigments.

Carotenoids of Elberta peaches. An investigation similar to the above was carried out on fresh, ripe Elberta peaches. The pigment mixture was found to be qualitatively quite similar to that for cling peaches. The 5,8-epoxides were obtained in relatively much greater quantities than in cling peaches. This indicates that the 5,6-epoxide–5,8-epoxide isomerization may proceed to a considerable extent in the ripening of acid fruits, especially when the fruit becomes soft.

SUMMARY

The carotenoids in cling peaches have been fractionated by the successive application of countercurrent distribution and chromatography. The pigment mixture was found to be quite complex and was predominantly xanthophylls, most of which occurred in the fruit as esters. The principal carotenoid constituents were violaxanthin, cryptoxanthin, β-carotene, phytoene, and an apparently previously undescribed xanthophyll for which the name "persicaxanthin" is proposed. Less abundant carotenoids were phytofluene, zeta-carotene, lutein, zeaxanthin, antheraxanthin, mutatoxanthins, luteoxanthins, trollixanthin-like polyols, and "persicachromes a and b." A number of minor constituents were also found, including several apparently undescribed carotenoids.

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LITERATURE CITED

1. Curl, A. L. On the structure of "hydroxy-α-carotene" from orange juice. *Food Research*, **21**, 689 (1956).
2. Curl, A. L. Application of countercurrent distribution to Valencia orange juice carotenoids. *J. Agr. Food Chem.*, **1**, 456 (1953).
3. Curl, A. L. and Bailey, G. F. The state of combination of the carotenoids of Valencia orange juice. *Food Research*, **20**, 371 (1955).
4. Curl, A. L., and Bailey, G. F. The carotenoids of Ruby Red grapefruit. *Food Research*, **22**, 63 (1957).
5. Curl, A. L., and Bailey, G. F. Polyoxygen carotenoids of Valencia orange juice. *J. Agr. Food Chem.*, **2**, 685 (1954).
6. Curl, A. L., and Bailey, G. F. Comparison of carotenoids of Valencia orange peel and pulp. *J. Agr. Food Chem.*, **4**, 156 (1956).
7. Curl, A. L., and Bailey, G. F. The carotenoids of tangerines. *J. Agr. Food Chem.*, **5**, 605 (1957).
8. Joyce, A. The effect of heat treatment on some plant carotenoids. Color in Foods. Advisory Board on Quartermaster Research and Development Committee on Foods, National Academy of Sciences, National Research Council, 148 (1954).
9. Karrer, P., and Jucker, E. Vorläufige Mitteilung über ein neues Carotinoid aus Orangenschalen: Citroxanthin. *Helv. Chim. Acta*, **27**, 1695 (1944).

10. KARRER, P., AND JUCKER, E. Die Konstitution des Citroxanthins. *Helv. Chim. Acta*, **30**, 536 (1947).
11. KARRER, P., KRAUSE-VOITH, E., AND STEINLIN, K. Ein neuer Blattfarbstoff: Xanthophyll epoxyd. *Helv. Chim. Acta*, **31**, 113 (1948).
12. KOE, B. K., AND ZECHMEISTER, L. Preparation and spectral characteristics of an all-trans and a cis-phytofluene. *Arch. Biochem. Biophys.*, **46**, 100 (1953).
13. KUHN, R., AND LEDERER, E. Über die Farbstoffe des Hummers (Astacus gammarus L.)und ihre Stammsubstanz, das Astacin. *Ber.*, **66**, 488 (1933).
14. MCCARTY, C. D., AND LESLIE, J. W. The carotenoids, amygdalin content, and titratable acidity of white- and yellow-fleshed peaches within a nearly isogenic line. *Proc. Am. Soc. Hort. Sci.*, **64**, 289 (1954).
15. MACKINNEY, G. Carotenoids of the peach. *Plant Physiol.*, **12**, 216 (1937).
16. MASE, Y., RABOURN, W. J., AND QUACKENBUSH, F. W. Carotene production by Penicillium sclerotiorum. *Arch. Biochem. Biophys.*, **68**, 150 (1957).
17. PETRACEK, F. J., AND ZECHMEISTER, L. Stereoisomeric phytofluenes. *J. Am. Chem. Soc.* **74**, 184 (1952).
18. RABOURN, W. J., AND QUACKENBUSH, F. W. The occurrence of phytoene in various plant materials. *Arch. Biochem. Biophys.*, **44**, 159 (1953).
19. SAPERSTEIN, S., AND STARR, M. P. The ketonic carotenoid canthaxanthin isolated from a color mutant of Corynebacterium michiganense. *Biochem J.*, **57**, 273 (1954).
20. THALER, H., AND SCHULTE, K. E. Die Carotinoide der gelben Pfirsiche. *Biochem. Z.*, **306**, 1 (1940).
21. TROMBLY, H. H., AND PORTER, J. W. Additional carotenes and a colorless polyene of Lycopersicon species and strains. *Arch. Biochem. Biophys.*, **43**, 443 (1953).
22. ZECHMEISTER, L., LEROSEN, A. L., SCHROEDER, W. A., POLGÁR, A., AND PAULING, L. Spectral characteristics and configuration of some stereoisomeric carotenoids including prolycopene and pro-γ-carotene. *J. Am. Chem. Soc.*, **65**, 1940 (1943).
23. ZECHMEISTER, L., AND PINCKARD, J. H. Some poly-cis lycopenes occurring in the fruit of Pyracantha. *J. Am. Chem. Soc.*, **69**, 1930 (1947).
24. ZECHMEISTER, L., AND POLGÁR, A. The carotenoid and provitamin A content of the watermelon. *J. Biol. Chem.*, **139**, 193 (1941).
25. ZECHMEISTER, L., AND SANDOVAL, A. The occurrence and estimation of phytofluene in plants. *Arch. Biochem.*, **8**, 425 (1945).

SPECTROPHOTOMETRIC DETERMINATION OF HYDROCYANIC ACID IN CANNED APRICOTS, CHERRIES, AND PRUNES

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The presence of the glucoside amygdalin in the pit of stone-fruits, such as cherries, peaches, and plums has been well established *(4)*. During the canning of whole, unpitted stone-fruits, the glucoside present in the pit is hydrolyzed into hydrocyanic acid, benzaldehyde, and glucose or other oligosaccharides. The harmful effect of hydrocyanic acid on health has been reported by various workers *(3, 4)*. Commercially, a large quantity of unpitted apricots, cherries, and prunes is canned either because of difficulties in removing the pits, or because of the improved aroma derived from the benzaldehyde formed. However, the literature contains little information on the quantity of hydrocyanic acid in such products. This work describes a spectrophotometric method for determining small quantities of hydrocyanic acid in canned, unpitted fruits, which is a modification of the method described by Aldridge *(1)* for determining microquantities of cyanide and thiocyanate in blood and other biological materials. This method involves steam distillation of hydrocyanic acid from the acidified sample, conversion of the cyanide in the distillate into cyanogen bromide, and spectrophotometric measurement of the optical density of the cyanide-benzidine complex at a wavelength of 520 mμ. The quantities of hydrocyanic acid present in canned unpitted apricots, cherries, and prunes are shown.

EXPERIMENTAL MATERIALS AND METHODS

Apricots. Blenheim apricots at canning maturity were washed in tap water, sorted for maturity according to skin color, and canned in plain No. 2½ cans with and without pitting. In the case of unpitted apricots, 12 fruits weighing 17 ± 0.5 oz. were packed in each can with 12 oz. of 40° Brix sucrose syrup; for pitted apricots, 19.5 oz. of fruit and 10.5 oz. of 40° Brix sucrose syrup were used. The cans were sealed under 15 inches of vacuum, cooked in a rotary cooker at 212° F. (100° C.) for 25 minutes or other specified intervals and then cooled in a rotary cooler for 5 minutes. The canned samples were stored at room temperature and analyzed for hydrocyanic acid content 4 months after canning.

Cherries. Bing cherries at canning maturity were canned in No. 2½ lacquered cans without pitting. Each can contained 18 oz. of washed and stemmed cherries and 12 oz. of 30° Brix sucrose syrup. Canning procedure was the same as that for apricots except that the cooking time was 20 minutes.

Cherry juice. Chatman cherries at canning maturity were washed and pulped through a 0.033-inch screen in a pilot-scale pulper to remove the seeds. The juice was filled into No. 2½ cans, sealed under 15 inches of vacuum, cooked in a rotary cooker at 212° F. (100° C.) for 37 minutes, and cooled for 5 minutes in a rotary cooler.

Prunes. French prunes at the firm-ripe stage were canned in plain No. 2½ cans, using 18 oz. of unpitted fruit and 11 oz. of distilled water for each can. Canning procedure was the same as that for apricots except that the canned product was cooked at 212° F. for 37 minutes.

Pressure test. Ripeness of 20 representative apricots was determined by the

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Magness-Taylor pressure tester with a pressure range of 0–10 lbs. and a plunger 5/16 inch in diameter.

Reagents. Sodium arsenite solution: 1.5 g. of reagent grade sodium arsenite dissolved in 100 ml. of distilled water.

Pyridine reagent: 50 ml. of reagent grade pyridine and 4 ml. of C.P. concentrated hydrochloric acid dissolved in enough distilled water to make 200 ml.

Benzidine hydrochloride solution: 1.43 g. of C.P. grade benzidine and 1.28 ml. of C.P. concentrated hydrochloric acid dissolved in enough water to make 100 ml. The mixture was centrifuged for 10 minutes to remove any insoluble substances.

Potassium cyanide stock solution: 0.241 g. of reagent grade potassium cyanide dissolved in 1 liter of distilled water. This stock solution when acidified has an equivalent concentration of 100 μg of hydrocyanic acid per ml.

Acetic acid. 0.2 N.

Saturated bromine water.

Steam distillation. The canned, whole stone-fruits were mixed with the syrup in a Waring blender for 30 seconds after the pits were removed. A 250-g. portion of the pulped sample was weighed and transferred quantitatively with a small amount of distilled water to a 500-ml. distillation flask *(5)*. Immediately after adding 25 ml. of 2 N sulfuric acid the flask was connected through glass joints with the condenser and the receiving flask. Steam was then applied to the distillation flask which was kept in a boiling water bath. The rate of distillation was so regulated that 35 ml. of distillate was collected in 20 to 30 minutes. The distillate was collected in a receiving flask containing 1 ml. of 0.1 N sodium hydroxide. Any uncondensed hydrocyanic acid was sucked under slight negative pressure through a sintered glass tube into a receiving tube containing 10 ml. of 0.05 N sodium hydroxide. The condenser and the receiving flask were chilled with ice-cold water, although tap water would have served the same purpose. The distillate in the receivers was combined and diluted with distilled water to 50 ml. in a volumetric flask.

Determination of hydrocyanic acid. A 10-ml. aliquot of the distillate was pipetted into a 50-ml. volumetric flask with glass stopper. Then 1.0 ml. of 0.2 N acetic acid and 2.0 ml. of saturated bromine water were added. Finally, 3.0 ml. of 1.5% sodium arsenite solution, 20 ml. of pyridine reagent, and 1.0 ml. of benzidine hydrochloride solution were added to the mixture in that order. The whole procedure was carried out under a ventilated hood. A blank was run, using the same procedure and reagents, except that 250 ml. of distilled water was used instead of the sample for steam distillation. The sample was allowed to stand at room temperature for 30 minutes during which an orange to red color was developed. The absorption at 520 mμ was measured in a Beckman DU spectrophotometer. The blank sample was set at 100 per cent transmission. The samples were analyzed in sequence, so that for each sample the time from the addition of benzidine to the spectrophotometric determination was the same.

RESULTS AND DISCUSSION

Absorption spectra of cyanide-benzidine complex. The absorption spectra for the cyanide-benzidine complex at hydrocyanic acid concentrations of 5, 10, and 20 μg per 10 ml. of sample respectively are shown in Figure 1. Since maximum optical density occurs at 520 mμ, it is advisable to measure the absorption at this wave length. Although Aldridge *(1)* recommended the use of a blue filter for colorimetric determination of cyanide in blood and other biological materials, it was found unsuitable for canned stone-fruits. This can easily be explained by the appearance in Figure 1 of a second absorption band between wave lengths of 400 and 450 mμ when the steam distillate from canned unpitted apricots was used. It was found that the Klett-Summerson photoelectric colorimeter could also be used with a green filter (No. 54) to determine the quantity of cyanide-benzidine complex. The results were comparable to those from Beckman DU at 520 mμ.

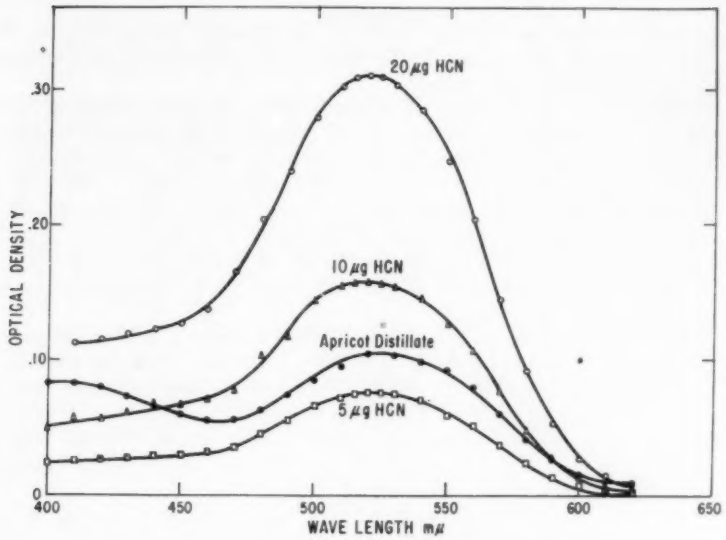

Figure 1. Absorption spectra of cyanide-benzidine complex.

The standard curve for the determination of hydrocyanic acid as cyanide-benzidine complex at 520 mμ in the concentration range of 0 to 20 μg of hydrocyanic acid per 10 ml. of sample or distillate followed the Beer-Lambert law. A straight line was obtained when the optical densities were plotted against the concentration. The reproducibility of the determination falls within 5%.

Sodium picrate test for hydrocyanic acid. In order to obtain additional evidence for the presence of hydrocyanic acid in canned unpitted apricots and cherries, the alkaline-sodium-picrate paper method described by the Association of Official Agricultural Chemists *(2)* was tried. The samples were pulped and steam-distilled as described previously. The distillate was diluted to 50 ml. and treated with 5 ml. of 2 N sulfuric acid in a stoppered Erlenmeyer flask in which the alkaline sodium picrate paper was hung above the sample. The presence of hydrocyanic acid in both samples was demonstrated by the change in color of the picrate paper from yellow to orange. It is interesting to note, however, that canned apricot halves did not give a positive test with the sodium picrate paper. This indicates that the glucoside amygdalin was present only in the kernel (embryo) of the pit, not in the flesh (mesocarp) of this fruit.

Influence of maturity and cooking time. The hydrocyanic acid contents of whole unpitted Blenheim apricots canned at 1.6 and 0.3 lbs. average pressure were found to be 0.13 p.p.m. for both samples. Thus, the maturity of the fresh fruit seems to have no influence on the hydrocyanic acid content of the canned product. This might imply that the amygdalin content of the embryo does not change at different stages of maturity. In another set of samples of whole unpitted apricots that were canned in 40° Brix sucrose

syrup, and sterilized at 212° F. (100° C.) in a rotary cooker for 18, 25, 33, and 40 minutes, no apparent difference in the hydrocyanic acid content of the canned products was found.

Hydrocyanic acid content of canned whole apricots, cherries, and prunes. The hydrocyanic acid content of canned whole apricots, cherries, and prunes was determined spectrophotometrically by the cyanide-benzidine method as described above. The results are shown in Table 1. No hydrocyanic acid was found in canned apricot halves which were pitted before canning. This indicates either that the amygdalin in apricots is present only in the kernel or that the concentration of hydrocyanic acid present in the flesh is so low that it is not detectable by the cyanide-benzidine spectrophotometric method.

Canned cherry juice had a hydrocyanic acid content of 0.44 p.p.m. while canned whole cherries had a value of 0.048 p.p.m. This could be attributed to: (a) mechanical break of the cherry pits in the pulping operation, which facilitated diffusion of amygdalin into the juice; (b) a dilution factor was introduced when sucrose syrup was added to the whole cherries during canning; or (c) possible difference in hydrocyanic acid content between Chatman and Bing varieties of cherries.

It has been reported by Aldridge *(1)* that cyanide in whole blood, plasma, serum, pancreatic juice, and other protein-containing fluids may be determined directly from the sample after deproteinization by means of trichloroacetic acid. However, erratic results were obtained when this method was applied to canned whole apricots. This could be attributed either to the presence of some interfering substances in the sample or to the presence of soluble pectin which causes turbidity and error in the spectrophotometric determination. Steam distillation of the hydrocyanic acid into a receiver containing sodium hydroxide overcame this difficulty.

The official method for the determination of hydrocyanic acid in beans *(2)* was based on precipitation of the cyanide in the steam distillate as silver salt, followed by titration of the excess silver ions with 0.02 N potassium thiocyanate, using ferric alum as an indicator. For canned stonefruits which are rather low in hydrocyanic acid content, the spectrophotometric cyanide-benzidine method described in this report seems to be more sensitive than the titration method.

Assuming that the lethal dosage of hydrocyanic acid for an average adult is 65 mg. *(4)*, the amount of this material found in canned apricots, cherries, and prunes is rather small.

TABLE 1

Hydrocyanic acid content of canned apricots, cherries, and prunes

Canned fruits	Variety	Maturity of fresh fruit	Hydrocyanic acid content, fruit and syrup combined, p.p.m. on wet basis
Apricots, whole, with pits	Blenheim	Normal	0.13
Apricots, halves, without pits	Blenheim	Normal	0
Cherries, whole, with pits	Bing	Firm-ripe	0.048
Cherry juice	Chatman	Firm-ripe	0.44
Prunes, whole, with pits	French	Firm-ripe	0.012

SUMMARY

A spectrophotometric method was adapted for the quantitative determination of small quantities of hydrocyanic acid in canned whole apricots, cherries, and prunes. The method involved steam distillation of the hydrocyanic acid from the sample into a receiver containing dilute sodium hydroxide. The distillate was analyzed for hydrocyanic acid content after acidification with acetic acid, conversion of the cyanide into cyanogen bromide, and spectrophotometric measurement of the optical density of the cyanide-benzidine complex at a wave length of 520 mμ.

This procedure has been applied to the determination of hydrocyanic acid in canned stone-fruits. The concentrations of hydrocyanic acid in samples of canned whole apricots, cherries, and prunes were found to be 0.13, 0.048, and 0.012 p.p.m., respectively. The sample of canned cherry juice had a hydrocyanic acid content of 0.44 p.p.m. In the case of canned apricots halves which were pitted prior to canning, no hydrocyanic acid was found. Thus amygdalin, the glucoside responsible for the formation of hydrocyanic acid during canning of whole stone-fruits, is present only in the pit, and not in the flesh of apricot fruit. Maturity of the fresh fruit and time of cooking during canning have no significant influence on the hydrocyanic acid content of the canned product. Assuming that the lethal dosage of hydrocyanic acid for an average adult is 65 mg., the amount of this material found in canned whole apricots, cherries, and prunes is rather small.

LITERATURE CITED

1. Aldridge, W. N. A new method for the estimation of micro quantities of cyanide and thiocyanate. *Analyst*, **69**, *262* (1944).
2. Association of Official Agricultural Chemists. *Official and Tentative Methods of Analysis*. 8th ed. 1955. p. 379. Washington, D.C.
3. Bamford, F. *Poisons, Their Isolation and Identification*. 1951. The Blakiston Co., Philadelphia, Pennsylvania.
4. Blyth, A. W., and Blyth, M. W. *Poisons, Their Effects and Detection*. 1920. Charles Griffin & Co., Ltd. London.
5. Luh, B. S., Leonard, S. J., Patel, D. S., and Claypool, L. L. Volatile reducing substances in canned Bartlett pears. *Food Technol.*, **9**, 639 (1955).

RATE OF MINERAL REMOVAL FROM MILK BY ION EXCHANGE[a,b]

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The importance of salt balance in the heat coagulation of fresh and evaporated milk was brought to the attention of the dairy industry by Sommer *(15)* almost 36 years ago. Calcium has been shown to be the controlling ion in the milk salt system primarily responsible for increasing heat instability of proteins due to coagulation of the calcium phosphate-calcium caseinate complex. In recent years ion exchange techniques have found application in the food processing and other industries. In the dairy industry ion exchange has been used to reduce calcium levels in evaporated milk *(4)* and to convert casein into a soluble alkali-metal caseinate of reduced ash content *(5)*.

Kunin *(11)* and Nachod *(13)* have shown that the strength of binding by exchangers increases with atomic weight and valence. The order of absorption for alkaline earth metals is $Ba^{++} > Sr^{++} > Ca^{++} > Mg^{++}$ followed by the monovalent alkali metals $Cs^{+} > Rb^{+} > K^{+} > Na^{+} > Li^{+}$. In our previous publications *(1,2)* it has been demonstrated that removal of calcium resulted in decreasing the tendency of the milk proteins to sediment under the high gravitational force of centrifugation *(1)* and in changes in opacity and flavor characteristics *(2)*.

The data presented in this paper are a result of experiments to determine the rates of removal of calcium and other ions from milk by resinous ion exchangers. Partial and/or complete demineralization of the pH sensitive dairy and other products is feasible when weakly acidic cation exchangers and weakly basic anion exchangers are employed in mixed bed columns.

The charts and nomographs presented here were found useful and time-saving in estimating the mineral levels in milk. It is hoped that with the increasing importance of ion exchange as a chemical engineering unit process, further applications in food technology will be facilitated by this material.

EXPERIMENTAL

Unless otherwise specified all demineralization procedures on fluid whole and nonfat milk were carried out in glass columns containing a mixture of weakly basic anion exchanger (Amberlite IR-45)[c] and a weakly acidic cation exchanger (Amberlite IR-50)[c] in the ratio of 3:2 volumes. Columns were charged with care to prevent channeling and air entrapment. Detailed description appears elsewhere *(1,2)*. Glass columns of 0.75, 1.5, 2.5 and 3.5 inches inside diameter were used in these studies. The columns were 48 inches high and were charged with the resin mixture to heights 10, 20, 30

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[b] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 28, 1958.

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and 40 inches. Fluid whole milk was passed downflow through the columns. The milk was kept cool and the process continued until milk equivalent to five volumes of resin was collected. It was experimentally determined that the degree of demineralization remained constant for volumes of milk not exceeding five volumes of resin in the column. The degree of demineralization decreased for successive 5-volume quantities of milk passed through the column progressively up to a total of 25 volumes of milk.

TABLE 1

Relation of milk volume to per cent decalcification (column 1.5-inch diameter, volume resin 500 ml.)

Milk x resin volume	I		II	
	Ca removed	Decrease	Ca removed	Decrease
	%	%	%	%
5	85.0	0	40.0	0
10	83.5	1.8	39.5	1.3
15	82.0	3.8	39.0	2.5
20	80.0	5.9	38.0	5.0
25	77.0	9.4	37.0	7.5

All analytical data for the rate of demineralization were obtained from thoroughly blended ion-exchanged milks consisting of the first 5 volumes drawn. Calcium was determined by precipitating calcium oxalate by means of urea hydrolysis *(8)*. Magnesium determinations were made colorimetrically using titan yellow *(12)*. Phosphorus was determined colorimetrically as molybdivanado-phosphoric acid *(10)*. Platinic chloride precipitation method was used to determine potassium *(14)*. Sodium was determined by zinc uranyl acetate reagent *(9)*.

The per cent removal of the various ions from milk was calculated from the weight per cent of each ion in exchanged milk to that of nonexchanged milk. The results shown in Figure 1 are mean values of several determinations of each ion and represent analytical data obtained over a period of several years on market milk samples. The

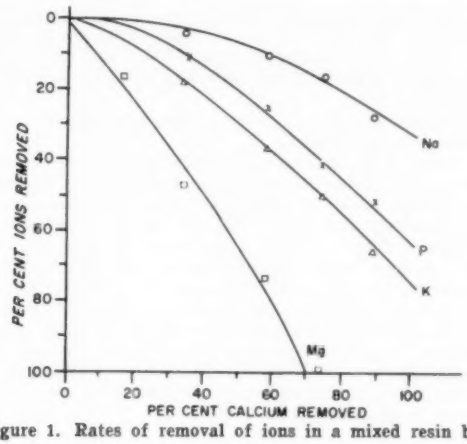

Figure 1. Rates of removal of ions in a mixed resin bed.

milligram weights of these ions in 100 ml. of whole non-ion exchange milk were: Ca—112.5, Mg—16.9, Na—50.6, K—157.7, P—85.6. Estimation of the degree of ion exchange using Figure 1 data checked within ±5% of the determined values.

The order of removal of ions shown in Figure 1 was $Mg^{++} > Ca^{++} > K^{+} > P > Na^{+}$. Calculated on mol fraction basis this order changed to $Ca^{++} > Mg^{++} > K^{+} > P > Na^{+}$.

On weight per cent basis all magnesium was exchanged when 30% of calcium, 55% of potassium, 65% of phosphorus and 85% of sodium remained. On mol fraction basis more than twice as much calcium was absorbed by the resin as of magnesium. For the alkali metals the quantity of potassium absorbed was more than six times that of sodium ion.

Further examination of Figure 1 indicates that levels of ion can be estimated in terms of known levels of calcium in ion-exchanged milk. It can be seen from Figure 1 that Mg^{++} ion is removed at the fastest rate, while Na^{+} ion is removed at the slowest rate. Experimentally, this process was carried out until the milk was completely demineralized, as determined by the total absence of any ash. Thus, using the mixed bed resinous ion exchange process described, completely demineralized milk of pH 6.6 to 6.8 was obtained. This product offers posibilities of developing milk completely free of sodium which could be used in low sodium diets or preparing a milk of any desired mineral composition.

Part 2—Ion exchange removal of calcium from milk. Demineralization of milk was studied in a column 1.5 inches in diameter and the data for ion exchange under varying conditions of resin height and rates of downflow were obtained. For each resin bed shown in Figure 2 five different rates of milk downflow were studied.

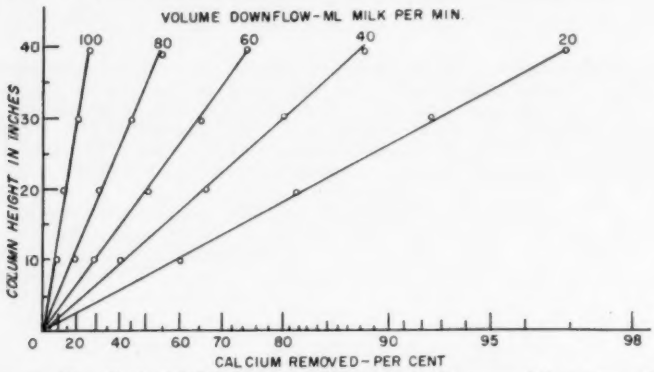

Figure 2. The effect of height and downflow rate on Ca removal in 1.5 in. column.

Each of the 5 curves represents a constant downflow rate for a total volume of 5 times the resin volume in the column.

The data in Figure 3 in addition to variable column height and milk downflow rate show variable column inside diameter. The 4 series of curves under A, B, C, D, represent each a different series of columns as previously described. The volume of milk collected for each variable studied did not exceed 5 volumes of resin used in the column. In Figures 2 and 3 scales were chosen to produce straight lines. It is thus apparent that calcium varies logarithmically with column height as shown in Figure 2 and geometrically with column diameter as shown in Figure 3.

The data presented in this paper cover a wider range of column diameters, volumes of resin and downflow rates than would be required for the usual laboratory demineralization determinations. It was experimentally determined that satisfactory results for milk demineralizations were obtained when a column of 1.5 inches inside diameter with resin bed 20 inches high was used and when the volume of milk was from five to 25 volumes that of resin in the column.

CONSTRUCTION AND USE OF NOMOGRAPHS

To facilitate the use of data presented in Figure 2 nomographs were constructed and are shown in Figures 4 and 5, respectively.

Construction. The nomograph shown in Figure 4 was designed for use when the inside diameter of the ion exchange column was constant at

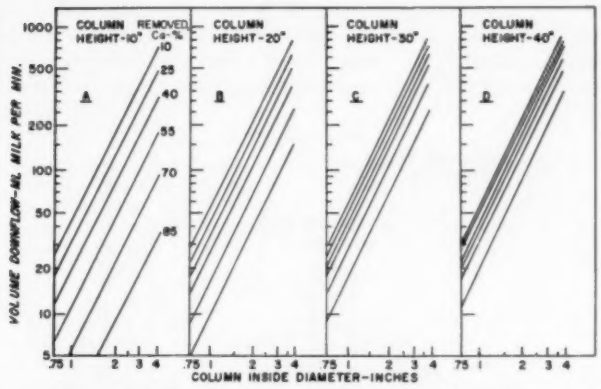

Figure 3. A nomograph for height, downflow rate and Ca removal in 1.5 in. column of monobed ion exchange resins.

1.5 inches. The nomograph shown in Figure 5 includes the column diameters as variables. The methods of construction of these nomographs were similar to those described in published works *(3, 6, 7)*.

Use of nomographs. This nomograph (Figure 4) is limited to a column of 1.5 inches inside diameter and a milk volume of five to 25 times the volume. For volumes beyond five times that of resin in the column, correction factors shown in Table 1 to estimate decrease of demineralization per cent were used.

Illustrative example. To remove 35% of calcium from milk equal to 10 volumes of resin in the column 25 inches high and 1.5 inches inside diameter, determine the downflow rate to give the desired decalcification. Place ruler on scale 3, marked PER CENT CALCIUM REMOVED,

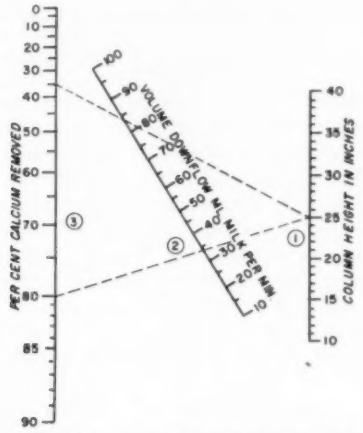

Figure 4. The effect of height, diameter and downflow rate on Ca removal.

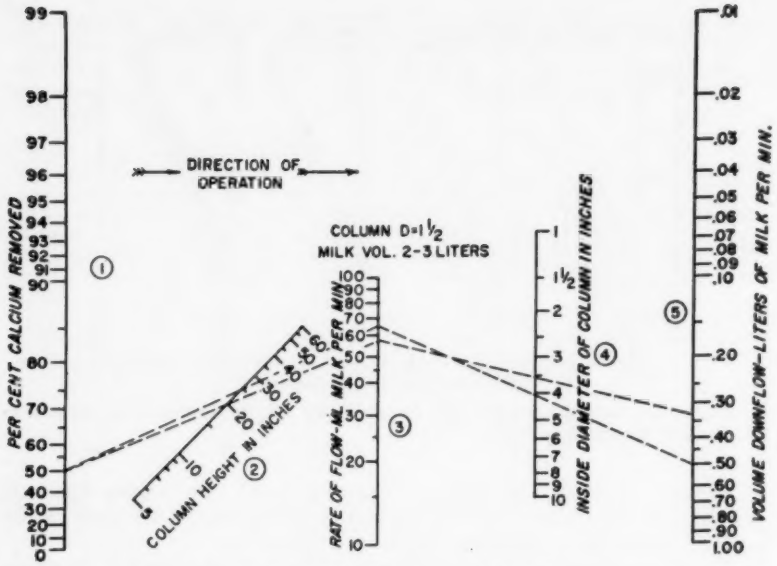

Figure 5. A nomograph for height, diameter and downflow rate on Ca removal in monobed ion exchange resins.

on 35 and the other end of the ruler on scale 1, marked COLUMN HEIGHT IN INCHES, *on 25*. Where the ruler crosses scale 2, the figure 83 represents milliliters of milk per minute of flow in a similar manner.

The nomograph shown in Figure 5 was constructed with a 1.5-inch column as a reference variable. Thus if column height and inside diameter are constants, either the downflow rate or per cent calcium removed may be determined if one or the other is known. The mechanics of operation of this nomograph are similar to that shown in Figure 4.

SUMMARY

Quantitative relations for the rates of removal of the major mineral ions from milk by mixed bed ion exchange system have been established. In a mixed bed of three parts of weakly basic anion exchanger and two parts of weakly acidic cation exchanger, magnesium, calcium, potassium, phosphorus and sodium ions were removed at rates decreasing in that order when calculated on weight per cent basis of these minerals in milk. The order for magnesium and calcium was reversed when the basis of calculation was mol fraction. In completely decalcified milk, potassium, sodium and phosphate ions remained in 25%, 70% and 40% of initial concentrations, respectively. Magnesium was completely removed when 70% of the weight of calcium was removed. These results indicate general compliance with the mass law principles.

The effects on rates and degree of calcium removal from milk by means of resinous ion exchangers through columns of varying diameter,

resin bed, height and milk downflow rates are described. Procedures for employing ion exchange process are described using charts and nomographs. Illustrative examples are given. Nomographs are presented which permit estimation of the extent of decalcification to within ±5% over a wide range of process variables.

Acknowledgment

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LITERATURE CITED

1. Bishov, S. J., and Mitchell, J. H., Jr. Sedimentation of milk proteins as a function of certain ions. *Food Technol.*, **10**, 312–315 (1956).
2. Coleman, R. D., Bishov, S. J., and Mitchell, J. H., Jr. Effects of calcium removal by ion exchange on the properties of fluid milk. *Food Technol.*, **8**, 211–215 (1954).
3. Davis, D. S. *Empirical Equations and Nomography.* 1943. McGraw-Hill, New York, N. Y.
4. Garrett, O. F. Applications of ion exchange to processing of milk products. XIIth Inst. Dairy Congress, Sect. IIIa, Subject Ic, *4*, Stockholm (1949).
5. Cehrke, C. W., and Almy, E. F. The action of mineral ion exchange resin on certain milk constituents. *Science*, **110**, 556 (1949).
6. Haugh, R. R. Computer for quick processing conveyor calculations. *Food Eng.*, 102–5, Aug. (1957).
7. Hoelscher, R. P., *et al. Graphic Aids in Engineering Computations.* 1952. McGraw-Hill, New York, N. Y.
8. Ingols, R. S., and Murrey, P. E. Urea-hydrolysis for precipitating calcium oxalate. *Anal. Chem.*, **21**, 525–527 (1949).
9. Jacobs, M. B. *The Chemical Analysis of Foods and Food Products.* 2nd ed. 1949. pp. 767–768, D. Van Nostrand Co., New York, N. Y.
10. Kiston, R. E., and Mellon, M. B. Colorimetric determination of phosphorus as molybdivanado phosphoric acid. *Ind. Eng. and Chem., Anal. Ed.*, **16**, 379–383 (1944).
11. Kunin, R. Ion exchange. *Ind. Eng. Chem.*, **44**, 79 (1952).
12. Ludwig, E. E., and Johnson, C. R. Colorimetric determination of magnesium by titan yellow. *Ind. Eng. Chem., Anal. Ed.*, **14**, 895–897 (1942).
13. Nachod, F. C. *Ion Exchange Theory and Applications.* 1949. Academic Press, Inc., New York, N. Y.
14. Official Methods of Analysis (6.16, 6.17). 7th ed., 1950, Association of Official Agricultural Chemists, Washington, D. C.
15. Sommer, H. H., and Hart, E. B. The heat coagulation of milk. *J. Dairy Sci.*, **5**, 525 (1922).

VISUAL AND EATING PREFERENCES OF CONSUMER HOUSEHOLD PANEL FOR BEEF OF DIFFERENT GRADES[a]

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In recent years a number of investigations using consumer rather than laboratory panels have been made to determine preferences of consumers for beef of different grades. Since studies of this type for meat are of recent origin and since meat presents some technical problems different from those of other products, methodology is still in a formative stage. For the most part, previous investigations have been concerned with consumers' *visual* preferences for graded beef. Seltzer *(6)*, and Stevens *et al. (8)* used photographs of graded beef while Coles *(1)*, Lasley, Kiehl, and Brady *(3)*, and Rhodes, Kiehl, and Brady *(5)* used a display of graded beef to obtain consumers' visual preferences. In addition to obtaining their visual preferences, Lasley, Kiehl, and Brady *(3)* secured an eating evaluation of the beef preferred visually by consumers. Kiehl, Rhodes, and Brady *(2)* recently obtained *eating* preferences of a consumer household panel using paired graded steaks. Methodological developments in consumer preference studies for meat have been reviewed by Naumann, Rhodes, Brady, and Kiehl *(4)*.

The present investigation obtains both the *visual and eating* preferences of a consumer household panel for beef of different grades. The purpose of this investigation is to determine (a) the existence of visual and eating preferences of panel members for paired steaks of lower and higher grade, and (b) the ability of panel members to select visually the grade of steak providing them with the greater eating satisfaction.

MATERIALS AND METHODS

Animals. Sixteen Hereford weaner steers, approximately 10 months old, from the University of California's range herd were used in this study. Information concerning history, feed-lot performance, and carcass analysis of these animals[b] was summarized in an article by Simone, Clegg, and Carroll *(7)*. Pertinent to a consumer preference study is the fact that steers of controlled history were used. Steers were above average in uniformity of breeding, age, and quality; they differed primarily in degree of finish. This difference was obtained by dividing the steers into equal groups, one of which was fed a higher percentage concentrate than the other. At time of slaughter, the 8 carcasses of the group fed the higher concentrate were graded by a Federal inspector designating 7 as *choice* and 1 as *good*, and for the other group 3 as *good* and 5 as *commercial*.[c]

Short loin and sirloin wholesale cuts from both sides of the carcasses were chosen. At weekly intervals both halves of 2 carcasses from each group which had been aged 11 days in a local private packing house were delivered to the Animal Husbandry Department. On the day of delivery the carcasses were broken down into wholesale cuts, chilled, and then further broken down into retail steak cuts one inch thick. Standardized cutting methods were used so that the first steaks from the loin and

[a] This report on preferences of a consumer household panel is part of a three-year study undertaken by the Departments of Animal Husbandry, Food Science and Technology, and Home Economics of the University of California at Davis.

[b] Animals were identified as the Trial 3 group.

[c] Grades were based on standards in effect in the spring of 1956.

sirloin wholesale cuts were at the same anatomical position for both sides of each carcass. Cutting of all carcasses was done by the same butcher, a University employee.

On the day of delivery the steaks were paired, trimmed, coded, and distributed by members of the Home Economics staff. Steaks from short loin and sirloin cuts of both sides of the group of carcasses grading higher were paired with identical cuts from the lower-grading group. For the 8 pairs of carcasses the grade pairings were: 3 pairs of *choice* and *good*, 4 pairs of *choice* and *commercial*, and 1 pair of *good* and *commercial*. Steaks were paired according to cutting sequence and thus came from the same relative location in their respective cuts. Each pair was then trimmed to the extent necessary to eliminate outside fat as a possible grade-recognition factor. For identification, one steak of each pair was tagged with a metal clip; the tagged steak alternately represented the higher and the lower grade.

Consumer household panel. The panel was selected from households of academic and nonacademic University personnel and of business and professional residents of Davis. The panel, composed of adult members of each household, can be described as representing a cross section of Davis socio-economic groups. A total of 398 households containing 821 adult panel members participated in the study during May and June, 1956.

A one-page preference sheet was used to record the reactions of each panel member to the paired steaks. Both a visual preference for the uncooked steaks and an eating preference based on over-all quality were recorded. Specific eating preferences for tastiness, juiciness, and tenderness were also indicated.

Distribution was accomplished either by a member of the household calling for the steaks at the Home Economics building or by a staff member who delivered them to the household. *Choice* and *good* steaks were compared by 151 households, *choice* and *commercial* by 196, and *good* and *commercial* by 51. Panel members were unaware of the fact that any difference between the two steaks was one of grade.

The panel member's visual preference at the time the steaks were distributed was recorded. Comparable sides of the steak were displayed for visual selection. The panel member was instructed to follow the same procedure in obtaining visual evaluations from other members in the household.

The use of the metal tag for identification purposes was explained to the panel member, who was instructed to leave it in place during the experiment. He was also informed that steaks were to be broiled or fried either the same or the following day, that no seasonings other than salt were to be used, and, in evaluating the steaks, that comparable parts were to be compared. Data on the preference sheet were requested to be supplied independently by each panel member.

RESULTS AND DISCUSSION

Preference sheets were returned by all but 2 of the 821 consumers participating in the study.[4] Information was obtained concerning method of cooking, degree of doneness, grade of meat usually eaten as determined by stores from which meat was purchased, and number of times in a month steak was eaten by panel members. These variables, which showed no systematic relation to the visual and eating preferences of panel members, are not discussed.

Visual and eating preferences of all panel members are summarized by wholesale cut and grade in Table 1. Since preferences appeared not to be related to sex of panel members and since differences by side of wholesale cut also were not apparent, information is not presented separately for these factors. For the paired grades *choice* and *good*, and *choice and commercial*, steaks graded *choice* were preferred for the most part to those graded *good* or *commercial*. These preferences were consistently more pronounced for steaks from the short loin than from the sirloin cuts. A laboratory study by Simone, Carroll and Clegg *(7)* was conducted concurrently pairing the same

[4] The 2 consumers had been given a pair of good-commercial steaks to evaluate.

TABLE 1

Preferences for steaks by wholsale cut and paired grades

Paired grades	No. of responses	Visual preference			Eating preferences											
					Over-all			Taste			Tenderness			Juciness		
		Higher grade	Lower grade	None	Higher grade	Lower grade	None	Higher grade	Lower grade	None	Higher grade	Lower grade	None	Higher grade	Lower grade	None
Short Loin (%)																
Choice–good	153	54[2]	30[2]	16[2]	66[2]	19[2]	15[2]	58[2]	16[2]	26[2]	61[2]	19[2]	20[2]	54[2]	18[2]	28[2]
Choice–commercial	213	50[2]	33[2]	17[2]	75[2]	11[2]	14[2]	59[2]	12[2]	29[2]	69[2]	12[2]	19[2]	65[2]	14[2]	21[2]
Total	366	52[2]	31[2]	17[2]	71[2]	15[2]	15[2]	58[2]	14[2]	28[2]	66[2]	15[2]	19[2]	60[2]	16[2]	24[2]
Good–commercial	52	60	25	15	44	41	15	35	36	29	21	46	33	39	46	15
Sirloin (%)																
Choice–good	156	42	42	16	47	37	16	43	29	28	38	39	23	45	33	22
Choice–commercial	191	45	41	14	53[1]	37[1]	10[1]	47[1]	30[1]	23[1]	36	40	24	44	36	20
Total	347	44	41	15	50[1]	37[1]	13[1]	45[2]	30[2]	25[2]	37	40	23	44	35	21
Good–commercial	54	37	54	9	21	57	22	24	50	26	11[1]	65[1]	24[1]	20	39	41

[1] Significant at the 5% level. Significance was determined by assigning half of the no-preference group to each of the two preference categories and calculating the appropriate value of X^2.

[2] Significant at the 1% level.

carcasses and using round and rib roast cuts. Results of the laboratory panel were in agreement with those of the consumer household panel; both panels preferred the higher to the lower grade.

For the short loin cut the preference for the *choice* irrespective of whether it was paired with the *good,* the *commercial,* or the combined *good* and *commercial* grades was significant at the 1% level for each preference factor evaluated. By comparison, for the sirloin cut the preference for *choice* was significant at the 5% level for only 2 factors; namely, over-all eating and taste. Furthermore, the over-all eating preference was significant at the 5% level only when the *choice* grade was paired with either the *commercial* or the combined *good* and *commercial* grades, whereas the taste preference was significant at the 5% level only when the *choice* was paired with the *commercial* grade. The taste preference, however, was also significant at the 1% level when the *choice* was paired with the combined *good* and *commercial* grades. It appears likely that for the short loin cut the high degree of significance indicated for each factor evaluated suggests easily recognizable differences in preference for different qualities of meat based upon grade; however, for the sirloin cut the degree of recognition of these differences was not as great.

For the paired grades *good* and *commercial* the tendency was for steaks which graded *commercial* to be preferred to those which graded *good,* and for this preference to be consistently more pronounced for steaks from the sirloin than from the short loin cuts. The visual and over-all eating preferences for steaks from the short loin were an exception to this tendency; these preferences were greater for steaks graded *good* than for those graded *commercial.* None of the preferences were significant at either the 1% or 5% level for the short loin, but for the sirloin cut the preference for the *commercial* grade was significant at the 5% level only for the tenderness factor.

It is generally assumed that a positive relationship exists between quality of steaks, as indicated by grade, and eating preferences of consumers. For a particular preference study this would imply that a more pronounced preference should exist for *choice* graded steaks when they are paired with *commercial* than with *good,* and that for paired steaks of *good* and *commercial* grade a more pronounced preference should exist for the former than for the latter.

In this preference study, for steaks from the short loin cut there was a tendency for the over-all eating preferences and for those of the 3 specific qualities to be more pronounced for the *choice* when the *choice* and *commercial* rather than the *choice* and *good* grades were paired. On the other hand, for steaks from the sirloin cut the tendency was for only two of the eating preferences—namely, over-all and taste—to be more pronounced for the *choice* when it was paired with the *commercial* grade. These tendencies were, however, too slight to be considered indicative of real differences. When *good* and *commercial* steaks were paired, the eating preference which existed, for the most part, were for the *commercial*; they did not, however, approach significance except for tenderness in the sirloin cut at the 5% level.

These results should not necessarily be interpreted to mean that a continuous positive relationship does not exist between quality of steaks, as indicated by grade, and eating preferences of consumers. For a particular

study, differences in preference between grades would be expected to depend upon the degree of quality variation between grades. For example, a more pronounced preference for the *choice* should exist if the comparison were between steaks which graded *high choice* and *low good* than between those which graded *low choice* and *average good*.

One method used to indicate quality differences among animals is fat content of the carcasses. In the present study percentage figures of fat content of the carcasses were estimated from specific gravity measurements of a 7-rib section (6th through the 12th rib). The differences in average percentages of fat between grades were: for the 3 pairs of carcasses grading *choice* (40.1) and *good* (32.2), a difference of 7.9; for the 4 pairs of carcasses grading *choice* (38.4) and *top commercial* (29.6), a difference of 8.8. For the 3 pairs of carcasess grading *good* (32.2) and the 4 pairs grading *commercial* (29.6), a difference of 2.6 which is less than the usual within-grade variation in fat content of carcasses. In the present study, it is apparent that significant quality variations exist between the 2 groups of animals fed on different percentage concentrates, that is, between one group of animals grading *choice* and the other grading either *good* or *commercial*. Consequently, in the remainder of this report preferences for the *choice* and the combined *good* and *commercial* grades are compared.

In the one case where *good* and *commercial* carcasses were paired, the difference in fat content for *good* (32.5) and *commercial* (27.6) was 4.9. Since this difference is not greater than the usual within-grade variation in fat content, no additional information is presented for the good-commercial group.

Comparison of individual preferences. Visual and over-all eating preferences of panel members are compared in Table 2. More than half of the panel members indicated the same preference for both visual and over-all

TABLE 2

Comparison of panel member's visual and over-all eating prefernces by wholesale cut

Comparison	Short Loin		Sirloin	
	%		%	
Same visual and eating preference				
No grade preference	6		4	
Preferred good or commercial grade	7		23	
Preferred choice grade	41		29	
Subtotal		54		56
Opposite visual and eating preference				
Visual, good or commercial; eating, choice	21		15	
Visual, choice; eating, good or commercial	6		10	
Subtotal		27		25
Preference and no preference—visual and eating				
Visual, no grade preference; eating, preferred good or commercial grade	1		5	
Visual, no grade preference; eating, preferred choice grade	10		6	
Visual, preferred good or commercial grade; eating, no grade preference	3		3	
Visual, preferred choice grade; eating, no grade preference	5		5	
Subtotal		19		19
Total		100		100

eating qualities of their steaks. By comparison, a fourth of the group reversed their grade preference indicating an eating preference opposite from their visual preference. The remainder of the group indicated a preference for one of the qualities but had no preference for the other.

Although the relative frequency with which panel members selected the same visual and over-all eating preferences was similar for steaks from both loin and sirloin cuts, the grades which these preferences represented differed for the 2 cuts. For the short loin cut three-fourths of the identical preferences were for steaks graded choice. By comparison, for the sirloin cut about half of the identical preferences were for steaks graded choice and two-fifths for those graded good or commercial.

A similar relationship existed for panel members having opposite visual and over-all eating preferences. For the short loin cut three-fourths of the panel members indicated the *good* or *commercial* grades on the basis of appearance but selected the *choice* on the basis of eating; the remaining fourth reversed these grade preferences for the two qualities. For the sirloin cut, three-fifths indicated the lower grades for appearance but the higher grade for eating; the remaining two-fifths reversed these grade preferences.

For the group having a preference for one quality but no preference for the other, differences between the 2 wholesale cuts existed for those members having a no-appearance preference accompanied by an eating preference. For the short loin cut nine-tenths of the preferences were for the *choice;* for the sirloin cut they were about equally divided between the higher and lower grades.

Table 3, a rearrangement of the Table 2 data, emphasizes not only the extent to which the over-all eating valuation of each of the 3 preference groups was consistent with the visual evaluation, but also the nature of the change in eating evaluation which occurred when it differed from the visual

TABLE 3

Analysis of over-all eating preferences for each of the three visual preference groups by wholesale cut

Visual and eating preferences	Short loin % of visual	Sirloin % of visual
Visual—no grade preference[1]		
Eating—no grade preference	36	30
—preferred good or commercial grade	8	33
—preferred choice grade	56	37
Total	100	100
Visual—preferred good or commercial grade[2]		
Eating—no grade preference	10	8
—preferred good or commercial grade	23	55
—preferred choice grade	67	37
Total	100	100
Visual—preferred choice grade[3]		
Eating—no grade preference	10	11
—preferred good or commercial grade	12	22
—preferred choice grade	78	67
Total	100	100

[1] This group consists of all no visual grade preferences of Table 2.
[2] This group consists of all visual good or commercial preferences of Table 2.
[3] This group consists of all visual choice preferences of Table 2.

evaluation. The relative frequency with which each of the 3 visual preference groups indicated identical eating preferences differed for the 2 wholesale cuts. For the short loin cut approximately four-fifths of the group having a visual preference for the *choice* grade, one-fourth of the group visually preferring the *good* or *commercial,* and one-third of those visually preferring neither grade indicated an eating preference the same as their visual. By comparison, for the sirloin cut the proportions were approximately two-thirds, one-half, and one-third, respectively.

When the eating preferences differed from the visual, the resulting eating evaluations of the 3 groups again were dissimilar for the 2 wholesale cuts. For the group having no visual preference, about nine-tenths of those indicating an eating preference favored the choice grade over the lower grades for steaks from the short loin cut, whereas the preferences were about equally divided for steaks from the sirloin cut. For the group having a visual preference for the lower grades, slightly more than one-tenth of those changing their eating evaluation indicated a no-eating preference as contrasted with almost nine-tenths indicating a reversal of their visual evaluation in favor of the *choice* grade for steaks from the short loin cut; the reversal was indicated by four-fifths of those having steaks from the sirloin cut. On the other hand, for the group having a visual preference for the *choice* grade, those changing their eating evaluation were as likely to have no grade preference as to prefer the lower grades for steaks from the short loin cut, but for steaks from the sirloin cut they reversed their eating preference in favor of the lower grades twice as frequently as they indicated a no-grade preference.

From the information presented in Tables 2 and 3 it appears that about half of the panel members were unable to select visually the grade of steak which provided them with the greater eating satisfaction. It also appears that the panel members who visually preferred the *choice* grade were much more consistent in their eating preference than were those having no visual grade preference or those visually preferring the lower grades.

Reasons for visual preference. The reasons most frequently given by panel members to explain their visual preference between the paired steaks are given in Table 4. Characteristics most frequently mentioned were mar-

TABLE 4

Reasons most frequently listed by panel members to explain visual preferences

Reason [1]	Choice grade		Good or commercial grade	
	Short loin % listing	Sirloin % listing	Short loin % listing	Sirloin % listing
Color of lean	31	20	41	36
Marbling	56	38	18	17
Proportion of lean to fat	15	23	28	25
General appearance	14	19	13	13
Tenderness, juiciness, freshness	11	17	8	12
Grain, texture	6	11	9	12
Amount of bone, cartilage, gristle	7	13	11	14
Amount of aging	3	2	8	3
Other	4	6	4	4

[1] Panel members were not limited to one reason.

bling for the *choice* and color of lean for the *good* or *commercial* grades. Amount of external fat, a factor frequently listed in other investigations of visual preferences for beef, was not mentioned by panel members since the paired steaks had been trimmed to eliminate this factor. Rhodes, Kiehl, and Brady *(5)* obtained visual preferences for 2 groups of graded loin steaks. External fat had been uniformly trimmed on the special but not on the regular display group; the distribution of preferences for the 2 groups were similar. The investigators concluded that the influence of the special trim was limited and that other factors besides external fat were important determinants of visual preferences.

The highly subjective nature of the responses presented in Table 4 was evident when the specific descriptive terms used by panel members were examined. For the most part panel members were not familiar with the technical term marbling; they referred to streaks of white or of fat in the lean meat and described them as more even, finer, and better looking. The terms used to describe marbling suggested that the panel members had not been told or trained to take this characteristic into consideration, but rather that their eating experiences had indicated it was an important visual characteristic to be considered when selecting satisfactory steaks. For color of lean, terms such as brighter, lighter, redder, and richer were used. Since only about half of the panel members had the same visual and over-all eating preferences, it would appear either that the visual characteristics which they used were inadequate to indicate desirable eating qualities, or that the panel members were unable to determine adequately the extent to which these characteristics were present in a specific steak.

Comparison of husband-wife preferences. Visual and over-all eating preferences of husbands and wives are compared in Table 5. In approximately half of the households the same visual and eating preferences for steaks from the loin and sirloin cuts were selected by both husbands and wives. As in the case of individual panel members, the grades which these

TABLE 5

Comparison of visual and over-all eating preferences of husband-wife households by wholesale cut

Comparison	Short loin		Sirloin	
	%		%	
Same visual and eating preference	50		52	
Same visual, different eating preference	14		13	
Same eating, different visual preference	29		22	
Different visual and eating preference	7		13	
Total		100		100
Same visual, different eating preference				
Visual same as wife's eating preference	67		32	
Visual same as husband's eating preference	29		47	
Visual different from either's eating preference	5		21	
Total		100		100
Same eating, different visual preference				
Eating same as wife's visual preference	38		28	
Eating same as husband's visual preference	43		53	
Eating different from either's visual preference	19		19	
Total		100		100

preferences represented differed for the 2 wholesale cuts, and preferences for the *choice* grade were much more pronounced for steaks from the short loin than from the sirloin cuts.*

When preferences differed, husbands and wives tended to disagree about twice as frequently in their evaluation of the visual than of the over-all eating quality. As a result of physiological differences in the loin and sirloin cuts, it appears likely that visual selection would be more difficult and eating satisfaction more variable for steaks from the sirloin than from the loin cuts. For the sirloin cut it is interesting to note that when visual preferences were identical the husbands' eating preferences agreed more frequently with their visual preferences than did the wives', and when eating preferences were identical the husbands' visual preferences agreed with their eating preferences more frequently than did the wives'. Although the reasons given by both husbands and wives in explaining their visual preferences between the paired steaks were similar, husbands appeared to have somewhat more success in visually selecting the grade of steak which provided the household with the greater eating satisfaction.

Comparison of over-all and specific eating qualities. The specific eating quality preferences of taste, tenderness, and juiciness are compared with the over-all eating quality preference of panel members in Table 6. As is to be expected, the variability in responses is much greater for the 3 specific qualities than for the over-all eating quality.

For both wholesale cuts the proportions having identical preferences for the over-all quality and for either two or three of the specific qualities ranged from about one-half to nine-tenths of the panel members; the remainder had identical preferences for none or for one specific quality. For the short loin cut, identical preferences for the over-all quality and for all 3 specific qualities were indicated relatively more frequently by panel members preferring the *choice* than by either those preferring the lower grades or those having no grade preference. For the sirloin cut, however, the proportions for the 3 groups were similar.

For the groups having identical higher or lower grade preferences for the over-all quality and for two specific qualities, tenderness and juiciness were the combination of qualities mentioned relatively more frequently for steaks from the short loin cut. By comparison, the combination of qualities selected for steaks from the sirloin cut differed not only by grade but also by sex of panel members.

For groups having identical higher or lower grade preferences for the over-all quality and for one specific quality, taste was indicated relatively more frequently for steaks from both wholesale cuts. It is interesting to note that when males and females had identical preferences for the over-all quality and for one specific quality, males tended to select the specific quality of taste relatively more frequently than did females. On the other

* Identical preferences of husband and wife for some of the 9 possible combinations of visual and over-all eating qualities were as follows: for steaks from the short loin cut, 55%, both qualities *choice;* 3%, both qualities *good* or *commercial* grades; 15%, visual quality lower grades and eating quality *choice* grade; 4%, visual quality *choice* and eating quality lower grades; for steaks from the sirloin cut the percentages were 35, 27, 12, and 12, respectively.

TABLE 6

Comparison of over-all eating preference with three eating quality preferences by wholesale cut of animals and sex of panel members

Comparison	Short Loin		Sirloin	
	Males	Females	Males	Females
	%	%	%	%
A. No grade preference				
No grade preference for over-all eating	100	100	100	100
and none for three eating qualities	28	29	33	50
and none for two eating qualities	34	21	50	40
and none for one eating quality	21	33	13	10
and a grade preference for three eating qualities	17	17	4	0
and none for the two eating qualities	100	100	100	100
taste and tenderness	10	20	58	63
tenderness and juiciness	0	20	0	13
taste and juiciness	90	60	42	25
and none for the one eating quality	100	100	100	100
taste	67	50	67	50
tenderness	17	13	33	0
juiciness	17	38	0	50
B. Good or commercial grade preference				
Preferred good or commercial grade for over-all eating	100	100	100	100
and also for three eating qualities	29	34	40	49
and also for two eating qualities	43	25	37	33
and no preference for these grades for three eating qualities	29	41	21	16
and also for one eating quality	0	0	2	2
and also for the two eating qualities	100	100	100	100
taste and tenderness	22	13	30	59
tenderness and juiciness	44	63	35	23
taste and juiciness	33	25	35	18
and also for the one eating quality	100	100	100	100
taste	100	62	38	27
tenderness	0	31	38	64
juiciness	0	8	23	9
C. Choice grade preference				
Preferred choice grade for over-all eating	100	100	100	100
and also for three eating qualities	54	55	41	43
and also for two eating qualities	29	30	44	39
and also for one eating quality	15	15	15	16
and no preference for these grades for three eating qualities	2	1	0	1
and also for the two eating qualities	100	100	100	100
taste and tenderness	32	33	47	22
tenderness and juiciness	38	47	17	28
taste and juiciness	29	21	36	50
and also for the one eating quality	100	100	100	100
taste	59	43	75	47
tenderness	12	38	8	20
juiciness	29	19	17	33

hand, the specific quality of tenderness was indicated relatively more frequently by females than by males. It is also interesting that when males and females had identical preferences for the over-all quality and for one specific quality, they indicated tenderness and juiciness much more frequently for *choice* than for *good* and *commercial* graded steaks from the short loin cut. When, however, their evaluation was based on steaks from the sirloin cut, this relationship between the higher and lower grades tended to be reversed. From Table 6 it would appear that panel members differed not only in their evaluation of the 3 specific eating qualities but also in the importance attached to each of these qualities in determining their over-all eating evaluation of the paired steaks.

SUMMARY

Visual and eating preferences for beef of different grades were obtained from a consumer household panel by testing paired graded steaks under actual home conditions. Preferences of the panel were definitely related to grade. In general, steaks graded *choice* were preferred to those graded *good* or *commercial*, and preferences were consistently more pronounced for steaks from the short loin than from the sirloin wholesale cuts.

Approximately half of the panel members indicated different preferences for the visual and over-all eating qualities of the paired graded steaks. It would appear either that visual characteristics used by panel members were inadequate or that panel members when selecting steaks were unable to judge the extent to which these characteristics were present.

About three-fourths of the panel members had identical preferences for the over-all eating quality and for two or three of the specific eating qualities. The variability in reponses, however, was much greater for the specific eating qualities of taste, tenderness, and juiciness than for the over-all quality. The results suggest that panel members differed considerably in the relative importance which they attached to the specific qualities in terms of providing eating satisfaction.

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LITERATURE CITED

1. Coles, Jessie V. Household buyers choose beef. *California Agriculture*, 10, 3 (1956).
2. Kiehl, Elmer R., Rhodes, V. James, Brady, D. E., and Naumann, H. D. St. Louis consumers' eating preferences for beef loin steaks. Mo. Agr. Expt. Sta., *Research Bulletin 652* (1958).
3. Lasley, Fred G., Kiehl, Elmer R., and Brady, D. E. Consumer preference for beef in relation to finish. Mo. Agr. Expt. Sta., *Research Bulletin 580* (1955).
4. Naumann, H. D., Rhodes, V. J., Brady, D. E., and Kiehl, E. R. Discrimination techniques in meat acceptance studies. *Food Technol.*, 11, 123–125 (1957).
5. Rhodes, V. James, Kiehl, Elmer R., and Brady, D. E. Visual preferences for grades of retail beef cuts. Mo. Agr. Expt. Sta., *Research Bulletin 583* (1955).
6. Seltzer, R. E. Consumer preference for beef. Ariz. Agr. Expt. Sta., *Bulletin 267* (1955).
7. Simone, Marion, Carroll, Floyd, and Clegg, M. T. Effect of degree of finish on differences in quality factors of beef. *Food Research*, 23, 32–40 (1958).
8. Stevens, Ira M., Sargent, Frederic O., Thiessen, Emma J., Schoonover, Carroll, and Payne, Irene. Beef . . . consumer use and preferences. Colo. Agr. Expt. Sta., *Bulletin 495 S* (1956).

AN INTENSITY–RESPONSE METHOD FOR THE MEASUREMENT OF FLAVOR INTENSITY

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The subjective measurement of flavor intensity is required for the solution of both fundamental and applied problems in flavor research. Examples of investigations which require this subjective evaluation include the effect of additives upon the intensity of a given taste component and the measurement of a food in terms of the intensity of standard solutions. The more applied problems include product control in relationship to the intensity of the flavor of the product, measurement of intensity changes during food processing, and determining the results of flavor fixation and flavor concentration studies.

Application of experimental designs dependent upon the relationship of intensity of taste to frequency of a response for measuring flavor intensity has been limited, and in general has been restricted to determining threshold values *(2)*. The purpose of the paper is to determine further the value of these methods in the assay of flavor intensity and in related problems.

In the field of psychophysics the use of intensity-response designs is firmly established *(1)*. They have been called various names such as Frequency Methods, the Method of Right and Wrong Cases, and the Method of Constant Stimuli. The basis of these procedures is the use of a series of stimuli varying from that which approaches complete perception in a series of trials to that which approaches no perception. The threshold value is then defined as the intensity of stimulus perceived 50% of the time. The 50% point is determined either by interpolation or more satisfactorily by the application of statistics.

The responses in these methods are in the nature of "present or absent", "yes or no", "more or less intense", etc. The statistics required are therefore those necessary for the interpretation of "all-or-none" or quantal responses. Quantal response statistics have been highly developed to meet the needs of many biological problems. This is especially true in pharmacology where the bioassay of drugs demands the solution of dose-effect relationships. One of the more recent methods of evaluating dose-effect experiments is that of Litchfield and Wilcoxon *(3)*. This method has the advantage of relative simplicity and speed without great loss of accuracy in the evaluation of quantal responses. The application of the Litchfield-Wilcoxon method to taste testing was suggested by S. Harrison formerly of these laboratories, and this application has proved successful both for determining threshold values and for assaying flavor intensity.

EXPERIMENTAL

Presentation of samples. Each taster is presented with 5 pairs of samples and asked to select the sample of higher flavor intensity or if he believes any pair of samples to be of equal intensity to so indicate. One sample in each pair is a member of a

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geometric series of 5 concentrations of a given flavor; the second sample in each pair is the same flavor but of unknown intensity. Pairs of samples are presented in random order both as to the position of the samples to be assayed and the series of known concentrations. A typical presentation of samples would be:

(u)	(5)	(2)	(u)	(4)
(3)	(u)	(u)	(1)	(u)

where "u" is the sample whose intensity is unknown and 1 through 5 the series of concentrations known to the person in charge of testing but not to the tasters.

Tabulation and calculations of results. For each concentration in the series the percentage of judgments selected as greater than the unknown concentration is calculated. That is, for each concentration in the series the number of judgments greater than the unknown plus one half the judgments indicating the given concentration as being equal to the unknown is divided by the total number of judgments.

These percentages are then plotted against their corresponding concentrations on logarithmic-probability paper. The intersection of the best-fitted line through these points with the 50% line gives an assay of the flavor of unknown concentration. The application of the Litchfield-Wilcoxen method will give the limits of error for this assay.

Not only is it possible to determine concentration and its limits of error but it is also possible to obtain a measure of the sensitivity of the panel in obtaining this assay. The slope of the line is a direct measure of such sensitivity. The Litchfield-Wilcoxen method permits the calculation of such a slope function value and its limits of error.

The following example (Table 1) is offered to illustrate further the tabulation and calculations of results. In this example, taken from a series of tests to evaluate the method and to screen tasters, the "unknown" concentration was 98 mg. of a cherry flavor per liter of water. This "unknown" was compared by 50 tasters to a series of

TABLE 1

Tabulation and calculation of results

Concentration	Judgments compared to unknown		% Judgments greater than unknown
	More intense	Equal	
50 mg. per liter	2	13	17
70 mg. per liter	5	17	27
98 mg. per liter	11	24	46
137.2 mg. per liter	29	13	71
192.1 mg. per liter	40	6	86

concentrations of the same flavor ranging from 50 mg. per liter to 192.1 mg. per liter.

When the percentages of judgments greater than the unknown are plotted against their respective concentrations, Figure 1 is obtained. The intersection of the 50% judgment line with the line determined by the plotted points is 96 mg. per liter. That is, this panel of 50 unscreened tasters determined the "unknown" concentration of 98 mg. per liter to be 96 mg. per liter.

The 95% confidence limits of this measurement are determined by applying the Litchfield-Wilcoxon expressions:

$$\text{upper limit} = ED_{50} \times f_{ED_{50}}$$

$$\text{lower limit} = ED_{50}/f_{ED_{50}}$$

$$\text{Where } f_{ED_{50}} = S^{2.77/\sqrt{N'}}$$

$$\text{and S, the Slope Function, } = \frac{ED_{84}/ED_{50} + ED_{50}/ED_{16}}{2}$$

ED_{50}, ED_{16}, and ED_{84} are the expected Effective Doses at 50, 16 and 84% respectively and N' = the total number of judgments between the 16 and 84% expected effects.

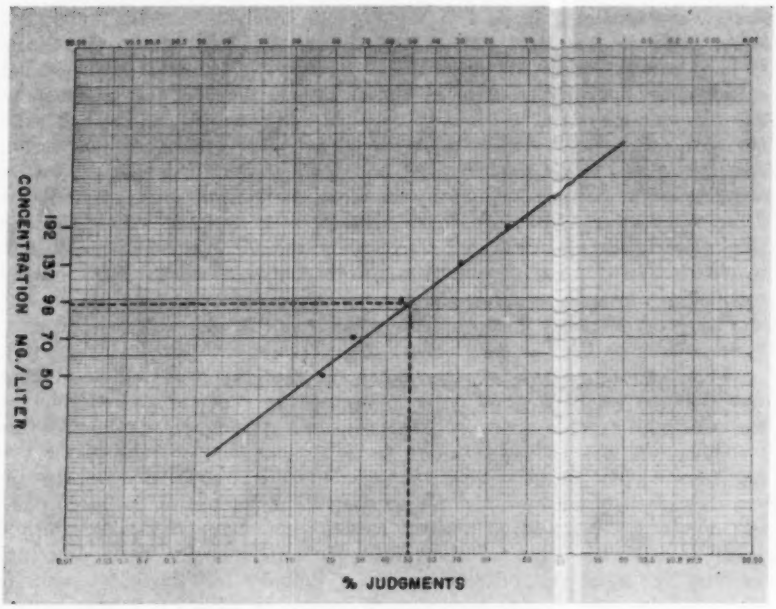

Figure 1. Assay of cherry flavor intensity.

In the above example we have:

$$S = \frac{180/96 + 96/51}{2} = 1.88$$

$$\text{and } f_{ED_{50}} = 1.88^{2.77/\sqrt{150}} = 1.15$$

Therefore, the upper limit = 96 × 1.15 = 110 and the lower limit = 96/1.15 = 83. Thus, this particular panel determined the "unknown" concentration with 95% confidence limits to be 96 (83 to 110) mg. per liter.

The actual calculation of these limits can be facilitated by the nomographs accompanying the Litchfield and Wilcoxon publication.

RESULTS AND DISCUSSION

Panel operation. The sample series is a logarithmic series both for statistical reasons and in conformity with the Weber-Fechner law. The Litchfield-Wilcoxon method is designed for balanced non-truncated systems and therefore it is necessary to select the series of concentrations to center about that of the unknown concentration. This can usually be accomplished by preliminary tasting by the experimenter or if a given series of concentrations produces unbalanced results, an adjusted series can be resubmitted to a panel and the combined results of the two tests utilized.

Originally, the presentation of samples was not in the form of the series of pairs of cups described above but involved a comparison of a randomized series of cups of increasing concentration to just a single cup of unknown concentration. It soon became evident that a modification of this procedure was necessary. After a few tests the tasters realized that

there were usually 2 levels less intense, one level about equal to, and 2 levels more intense than the control—the experimental design necessary for a proper application of the statistical procedure. Furthermore, in making a judgment involving a series of concentrations there is the tendency to select the middle of the series as being equal to the control, the tasters were not only influenced by their prior knowledge of the experimental design but also by this error of a "central tendency." A test in which the lowest member of the series was actually equal to the control demonstrated that these two factors had a significant effect upon the panel's results. These effects were also demonstrated by dividing the flavor matching test into two parts. In this manner the first half of the test had the least intense member of the series equal to the control, while the second half of the test had the most intense members of the series equal to the control. The combined results of the half tests failed to plot as the required straight line.

In order to overcome these sources of error the presentation of cups was changed to the method outlined in the experimental section, that is, the presentation of a series of pairs of cups. When this modified flavor matching test was divided into two parts, the combined results of the two half tests do plot as the required straight line. A possible objection to this modification may be the error due to adaptation because of the large number of samples involved. However, a test with cherry flavor, in which 6 pairs of flavors with a constant difference in intensity were presented revealed no significant differences in the judgment of the latter pairs of flavor as compared to the first pairs of flavor. In addition, a large variety of samples have been evaluated in this manner in these laboratories and only in the case of extremely bitter materials has it been found necessary to limit the presentation of cups to less than 5 pairs.

Applications of the method. The example given in the experimental section serves to illustrate two important applications of the method. These are (a) the assay of flavor intensity and (b) the measurement of a given panel's sensitivity. In a similar manner it is possible to obtain a measurement of an individual panel member's sensitivity. All that is required is to obtain repeated judgments at each of a series of concentrations for a given panel member.

The calculation of the sensitivity together with its limits of error for a panel or for a given panel member makes possible a comparison of sensitivities. The comparison of panel members' sensitivities can serve as a basis for ranking tasters. This can be accomplished by either directly comparing judge's sensitivities following repeated judgments or by comparing a given judge's sensitivity to that of the entire panel. Of course, it is also possible to make comparisons of the slopes of subsets of judges from a given panel or to compare the sensitivity of a panel on a given day to previous performances.

The comparison of panel sensitivities also permits a study of the effect of a change in panel sensitivity with a change in experimental conditions. An example of a study of this type was the change in a panel's sensitivity with a change in tasting media. The greatest portion of a series of intensity evaluations upon a cherry flavor were made in plain spring water. The possibility of increasing panel sensitivity by using the more pleasant base produced upon the addition of sugar and citric acid to the spring

water was investigated. The combined results of 47 tasters over a series of 23 tests in which the same flavor had been evaluated in plain spring water was plotted. In this manner a sensitivity (slope function) 1.61 (1.56 to 1.66) was obtained. However, the addition of the sugar and citric acid served only to confuse the panel, as there was a significant loss in sensitivity to 2.46 (1.64 to 3.69) for 45 tasters in a single test.

The determination of the 95% confidence limits for these slope functions and the fact that this loss in sensitivity is statistically significant again is established by applying the Litchfield-Wilcoxon analysis.

95% confidence limits:

$$\text{upper limit} = S \times f_s$$
$$\text{lower limit} = S/f_s$$

Where S is the Slope Function as previously defined and

$$\log f_s = \frac{10(K-1)}{K\sqrt{N'}} \cdot \frac{1.1(\log S)^2}{\log R}$$

The definitions for the new terms R and K are:

R = the ratio of largest/smallest concentration plotted.
K = the number of concentrations plotted.

Thus, for the combined results of the 47 tasters with a sensitivity (S) of 1.61 we have:

$$\log f_s = \frac{10(5-1)}{5\sqrt{3 \times 47 \times 23}} \cdot \frac{1.1(\log 1.61)^2}{\log (192.1/50)}$$

$$\text{or } f_s = 1.03$$
$$\text{upper limit} = 1.61 \times 1.03 = 1.66$$
$$\text{lower limit} = 1.61/1.03 = 1.56$$

For the panel matching flavors in the sugar and citric acid medium with a sensitivity of 2.46, the f_s value of 1.50 was calculated in a similar manner.

These two sensitivities are significantly different (19/20 probability) if their slope function ratio, S.R., exceeds the factor, $f_{S.R.}$

That is $S.R. = S_1/S_2$ must exceed

$$f_{S.R.} = \text{antilog}\sqrt{(\log f_{s_1})^2 + (\log f_{s_2})^2}$$

Therefore, in the above comparison of sensitivities we have

$$S.R. = 2.46/1.61 = 1.53$$
$$\text{and } f_{S.R.} = \text{antilog}\sqrt{(\log 1.50)^2 + (\log 1.03)^2}$$
$$f_{S.R.} = 1.50$$

Since 1.53, the slope function ratio, exceeds 1.50, the factor $f_{S.R.}$, the two sensitivities are significantly different.

The Litchfield and Wilcoxon publication again provides nomographs to facilitate these calculations.

Note, however, that this comparison was based upon a large number of judgments for the determinations made in plain spring water. This of course resulted in a more precise determination of the panel's sensitivity under

these conditions and in turn made possible the significant comparison to the second set of conditions. In another comparison the panel sensitivity for flavor-matching orange flavor was 2.19 (1.40 to 3.42) and for lemon-lime 1.65 (1.38 to 1.98). With panels of 31 and 35 individuals respectively, the measurements of panel sensitivity were not precise enough to state that the differences in sensitivity are statistically significant at a meaningful confidence level.

Both in the assay of flavor intensity and for the measurement of sensitivity it is always possible to improve the precision—that is, to narrow the confidence limits of these measurements. This can be accomplished, as suggested above, by an increase in the number of judgments either by an increase in panel size or the number of concentrations to be compared and by an improvement in panel sensitivity. Thus, any method to improve panel motivation, panel training, tasting conditions, or any method to select the more sensitive judges will yield a lower value for S, the Slope Function, and therefore, narrower confidence values.

CONCLUSION

The application of an intensity-response design to the measurement of flavor intensity has proved of value in the laboratories. This method not only appears to be psychologically sound but offers decided statistical advantages. In addition to establishing a flavor level in the form of grams of flavor per volume of solution together with the limits of error for the measurement, the method provides a determination of the panel's sensitivity or a given panel member's sensitivity together with the limits of error. This measurement of panel sensitivity provides a method of studying the effects of change in experimental conditions on panel performance, while the measurement of panel members' sensitivities is a method of ranking tasters.

Acknowledgment

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LITERATURE CITED

1. Guilford, J. P. *Psychometric Methods.* 2nd ed. 1954. McGraw-Hill, New York, p. 118.
2. Harrison, S., and Elder, L. W. Some applications of statistics to laboratory taste testing. *Food Technol.*, **4**, 434 (1950).
3. Litchfield, J. T., Jr., and Wilcoxon, F. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exptl. Therap.*, **96**, 99 (1949).

A NOTE ON A METHOD FOR THE EXTRACTION OF FREE FATTY ACIDS FROM LIPID MATERIAL[a]

The extraction of fatty acids from isolated plant lipid material is hampered by troublesome emulsions. It has long been known that the Röse-Gottlieb method *(2)*, which is employed for the quantitative estimation of fat in dairy products, avoids such emulsions by the use of ethyl alcohol in the extraction medium, which also contains equal volumes of ethyl ether and petroleum ether (b.p. up to 60° C.). Such a solvent mixture can be used to advantage for the elimination of the emulsion problem and excessive centrifugation in the extraction of free fatty acids from lipid material.

PROCEDURE

Weigh 5 g. of lipid sample into a 250 ml. Erlenmeyer flask. Transfer the sample to a 250 ml. separatory funnel with a solvent composed of 70 ml. of ethyl ether, 70 ml. of petroleum ether (b.p. up to 60° C.), and 25 ml. of 95% ethyl alcohol. Add 50 ml. of 1% solution of sodium carbonate, and shake vigorously for 30 seconds. Following the separation of the layers on standing, draw off the lower aqueous phase. To the ethereal solution remaining in the separatory funnel, add 5 ml. of 95% ethyl alcohol and 30 ml. of 1% sodium carbonate solution. Again vigorously shake for 30 seconds, and allow to stand until the layers completely separate. Draw off the lower aqueous phase as before, and combine with the first extraction. For the third extraction add 5 ml. of 95% ethyl alcohol and 20 ml. of 1% sodium carbonate solution, followed by vigorous shaking as previously described. Draw off the lower aqueous layer and pool with the previous aqueous extracts. A final extraction is made with 25 ml. of distilled water, and combined with the previous aqueous extracts. The ethereal layer contains the neutral fatty material, and may be reserved for further investigation, if desired.

Acidify the aqueous extract with 5 ml. of 10% sulfuric acid solution and return to a clean 250 ml. separatory funnel. Extract the fatty acids with 50 ml. of ethyl ether and petroleum ether mixture (1:1, v/v). Repeat this procedure 3 times. Pool the ether extracts and dry over anhydrous sodium sulfate. Remove the solvents by evaporation in vacuum. To determine the amount of isolated fatty acids, dry the above residue to constant weight over phosphorus pentoxide, weigh, and calculate the percentage recovery. To determine the percentage recovery by titration, dissolve the residue in 20 ml. of neutral ethyl alcohol and titrate with 0.01 N sodium hydroxide to a phenolphthalein end-point *(1)*.

RESULTS

A series of stearic acid samples ranging from 0 to 50 mg. by increments of 10 mg. were dissolved in ethyl ether, added to Erlenmeyer flasks and thoroughly mixed with 5 g. of lipid sample. Results obtained are presented in Table 1 and indicate that the method is highly satisfactory for the recovery of free fatty acids from lipid material. This is further indicated by the regression equation calculated to be $Y = 0.969X - 0.10$, where Y is the dependent variable, mg. fatty acid recovered, and X is the independent variable, mg. fatty acid added. The standard error of estimate for this equation was calculated to be ± 0.55 mg. of fatty acid (stearic acid). The average percentage of fatty acid recovered was 96.6%, both by weight and by titration.

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TABLE 1

Relationsip between the fatty acid recovered employing the extraction technique and the fatty acid added to lipid

Mg. stearic acid added	Mg. stearic acid recovered by titration	Per cent recovery	Mg. stearic acid recovered by weight	Per cent recovery
0	0	.....	0.2	.....
10.0	9.64	96.4	9.8	98.0
20.0	19.7	98.5	19.2	96.0
30.0	28.1	93.7	29.1	97.0
40.0	38.7	96.8	37.6	94.0
50.0	48.7	97.4	48.9	97.8

SUMMARY

A method is presented for the extraction of free fatty acids from lipid material. This method eliminates the troublesome emulsions formed during the extraction of soap solutions with ether.

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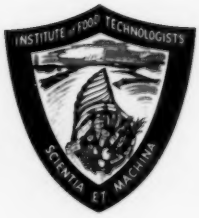
LITERATURE CITED

1. Association of Official Agricultural Chemists. *Official and Tentative Methods of Analysis.* 8th ed., 1955. p. 470. Washington, D.C.
2. GOTTLIEB, E. *Eine bequeme Methode zur Bestimmung von Fett in Milch. Landw. Vers. Sta.* **40**, 1–27 (1892).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